GENES ENCODING THE KEY ENZYMES FOR THE BACTERIAL DEGRADATION OF THE NATURAL NITRO COMPOUNDS 3-NITROTYROSINE AND 1-NITRO-2-PHENYLETHANE

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This thesis research is dedicated to my family. To my husband for encouraging me, to my mother for dedicating 18 months to helping me, and to my sons for giving me a better reason for accomplishing this feat.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

¼ TSA	¼ Strength tryptic soy agar
¼ TSB	¼ Strength tryptic soy broth
3NBA	3-Nitrobutanoic acid
3NPA	3-Nitropropionic acid
3NTyr	3-Nitrotyrosine
8F12	Fosmid containing *denA* from *Variovorax paradoxus* sp. JS171
BCA	Bicinchoninic acid
BLK (-N)	Bruhn-Lenke-Knackmuss minimal media (with nitrogen)
bphA1	Biphenyl dioxygenase, large α-subunit
bphA2	Biphenyl dioxygenase, small β-subunit
bphA3	Biphenyl dioxygenase, ferredoxin
bphA4	Biphenyl dioxygenase, reductase
BphD	Hydrolase involved with biphenyl degradation
C-C	Carbon-carbon
C-terminus	Carboxyl terminus, polypeptide
denA	Gene encoding 4-hydroxy-3-nitro-phenylacetate denitrase
Δ*denA*::JS669	Insertion mutation of the *denA* gene in *Variovorax* sp. JS669
DMF	N, N-Dimethylformamide
Δ*npeA1*::JS670	Insertion mutation of the *npeA1* gene in *Burkholderia* sp. JS670
dNTP	Deoxyribonucleotide triphosphate
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GC/MS	Gas chromatography-mass spectrometry
HNPA	4-Hydroxy-3-nitro-phenylacetate
HPC	Homoprotocatechuate
HPLC	High performance liquid chromatography
IPTG	Isopropyl b-D-1-thiogalactopyranoside
ISP	Iron sulfur protein
JS165	*Burkholderia* sp. JS165

JS171	*Variovorax paradoxus* sp. JS171
JS669	*Variovorax* sp. JS669
JS670	*Burkholderia* sp. JS670
LB	Luria-Bertani broth
MSA	Multiple sequence alignment
NOS	Nitric oxide synthase
NPB	2-Nitropropylbenzene
NPE	1-Nitro-2-phenylethane
npeA1	Gene encoding NPE dioxygenase, alpha subunit
npeD	Gene encoding hydrolase in NPE degradation pathway
N-terminus	Amino terminus, polypeptide
OD_{600}	Optical density, at 600 nanometers wavelength
ORF	Open reading frame
PCR	Polymerase Chain Reaction
pJS337	pBAD construct containing *denA* from *Variovorax paradoxus* sp. JS171
pJS338	pBAD construct containing *denA* from *Bordetella bronchiseptica* RB50
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
R	Un-described chemical structure
R^2	Correlation factor
SOC	Super optimal broth with catabolite repression
CAC	Citric Acid Cycle
TNT	2,4,6-trinitrotoluene
todC1	Gene encoding toluene dioxygenase, α-subunit

SUMMARY

Natural nitro compounds are produced by bacteria, fungi, plants and animals and exhibit diverse structures and biological functions. Little is known about the behavior of such compounds in natural ecosystems. The lack of accumulation in the biosphere implies that they are biodegraded. Although there has been significant research conducted to elucidate microbial strategies for biodegradation of synthetic nitro compounds, there have been only two pathways published detailing metabolism of natural nitro compounds. This research examines the genes that encode the key enzymes required for biodegradation of 3-nitrotyrosine and 1-nitro-2-phenylethane.

The biodegradation pathway for 3-nitrotyrosine has been described, however the gene encoding the key enzyme remained a mystery. Here we report the discovery of *denA*, which encodes a denitrase that is key to the biodegradation of 3-nitrotyrosine. *In silico* analysis of *denA* revealed that the encoded enzyme forms a novel clade among FAD monooxygenases. Furthermore, testing of a previously uncharacterized enzyme in the denitrase clade revealed that the related enzyme was also a denitrase. Thus, identification of *denA* enabled functional annotation of a previously unknown enzyme. Analysis of 16S rRNA phylogeny of bacterial isolates capable of 3-nitrotyrosine biodegradation revealed that the metabolism is widespread among diverse bacteria. Comparison of *denA*- encoded enzymes among 3-nitrotyrosine degrading bacteria revealed several conserved motifs. Additional comparison of the *denA*-encoded enzyme with other enzymes that catalyze denitration, dechlorination and defluorination permitted discovery of conserved residues among all three groups of enzymes. Some of the

conserved regions are shared among other FAD monooxygenases, whereas other conserved residues appear to be unique among enzymes that remove nitro-, chloro- and fluoro- groups by monooxygenation.

1-Nitro-2-phenylethane has been identified from several plants, yet the biodegradation of the compound remained a mystery. Here we report the degradation of 1-nitro-2-phenylethane, as studied through its analog 2-nitropropylbenzene. A combination of molecular and biochemical methods was used to elucidate the biodegradation pathway for the two compounds. Discovery of the metabolic pathway revealed a novel microbial strategy to use a *meta*-ring fission degradation pathway to cleave an undesirable side chain from an aromatic compound and use the remainder of the compound as a carbon and energy source. Two genes that encode enzymes in the biodegradation pathway were identified and both are deeply branched within their respective phylogenetic trees, indicating that both represent highly specialized microbial enzymes. Furthermore, it was discovered that microbial degradation of 1-nitro-2-phenylethane resulted in the production of 3-nitropropionic acid, a natural nitro toxin that inhibits succinate dehydrogenase and is responsible for livestock illness and death. This is the first report of bacterial production of 3-nitropropionic acid, and might represent a significant source of 3-nitropropionic acid in natural habitats.

The findings from these studies contribute to the overall understanding of microbial metabolism. Specifically, this research reveals genes that encode novel enzymes and strategies for the biodegradation of two natural nitro compounds. Furthermore, discovery of mechanisms for the biodegradation of such compounds reveals

novel microbial metabolic diversity and provides insight into the evolution of degradation pathways for synthetic compounds.

CHAPTER 1

INTRODUCTION

1.1 Overview of nitro compounds

Natural and synthetic nitro compounds are used widely as solvents, explosives, dyes, antibiotics, pesticides, fungicides and herbicides. Many nitro compounds are anthropogenic, however many are naturally produced (179) by both prokaryotic (1, 145) and eukaryotic organisms (10, 23, 57, 72, 101, 177). Biodegradation of synthetic nitro compounds has previously attracted much attention, and several reviews have been dedicated to such discussion (66, 119, 123, 157, 185, 189). Biodegradation of natural nitro compounds is less well studied, and few examples have been described (120, 142). Similarly, the ecological roles and impact of the natural nitro compounds are poorly understood.

Nitro compounds are often classified as primary pollutants due to toxicity and/or mutagenicity (107, 128). The chemical characteristics of nitroaromatic compounds are key to their recalcitrance towards degradation. Aromatic structures are stable due to their resonance structures and thermodynamic stability (93, 173). The nitro moiety is strongly electron withdrawing, which greatly impacts potential biodegradation of the compounds by helping to shield them from initial oxygenase attack (119, 123). The nitro group also increases the solubility of the aromatic compounds, making it relatively easy for them to diffuse and disperse in soil and water (185).

Studies of metabolism of both anthropogenic and natural nitro compounds are important for the understanding of the flux of such compounds in soil. Synthetic nitro compounds can be recalcitrant, although many are biodegradable by soil microbes (17, 31, 38, 53, 60, 66, 118, 136, 164, 185, 189). Much is known about biodegradation of synthetic nitroaromatic compounds, however there is little information about the metabolic diversity and catabolic capacity among microbes that degrade natural nitroaromatic compounds.

1.2 Thesis/Research Overview

Previous research has defined a diverse set of pathways for the biodegradation of synthetic nitro compounds and identified a wide spectrum of natural nitro compounds; such research led to questions as to how natural nitro compounds are metabolized and what purpose natural nitro compounds serve in ecosystems. The goal of this research is the discovery of mechanisms for the biodegradation for natural nitro compounds, which will reveal novel metabolic diversity, potential enzymes for green chemistries and insight about the evolution of pathways for degradation of synthetic nitro compounds. Chapter 1 is a literature review of the synthesis, biodegradation and putative ecological roles of nitro compounds. The roles of key metabolic enzymes are presented along with a discussion about gene discovery and evolution of metabolic pathways. In the chapters following the literature review, research into the biodegradation of two natural nitro compounds, 3-nitrotyrosine (3NTyr) and 1-nitro-2-phenylethane (NPE) is presented. The

research in chapters 2 and 3 aimed to reveal the gene that encodes the key enzyme required for 3NTyr metabolism, as well as demonstrate the phylogenetic diversity of bacteria capable of 3NTyr degradation. In chapter 2, experiments with *Variovorax* sp. JS669, a novel 3NTyr-degrading isolate, revealed the key gene (*denA*) and enzyme (4-hydroxy-3-nitro-phenylacetate denitrase, designated DenA) for the biodegradation of 3NTyr. Phylogenetic characterization of the enzyme revealed a novel subset of catabolic enzymes. DenA diversity among 3NTyr-degrading isolates is explored in chapter 3. Comparisons between 16S rRNA and DenA phylogeny of 3NTyr-degrading isolates provide insight into the evolution of DenA and the spectrum of 3NTyr-degrading microbes. The objective of the research presented in chapter 4 was to elucidate the NPE biodegradation pathway, and to identify the genes encoding key enzymes required for NPE metabolism. Research in chapter 4 describes the identification of the NPE biodegradation pathway, along with the genes that encode the dioxygenase α-subunit and hydrolase enzymes required for NPE metabolism in *Burkholderia* sp. JS670. The substrate range of the NPE degradation pathway is explored to determine the potential of the key NPE catabolic enzymes for the degradation of a variety of substituted aromatic compounds.

1.3 Overview of natural nitro compounds

Although natural nitro compounds are known to exist in multiple ecosystems (1, 23, 24, 180) their ecological significance, sources and flux are not well understood.

Previously believed to be rare, natural nitro compounds are continually being identified (Figure 1.1) (1, 45, 179). Interestingly, the natural nitro compounds do not accumulate or persist in natural ecosystems implying that there is a flux among organisms that produce the nitro compounds and organisms that are capable of transforming or mineralizing them. To date, few degradation pathways for natural nitro compounds have been described (120, 142) because most natural nitro compounds are studied only if they are known to be toxins or antibiotics (3, 4, 10, 15, 23, 140).

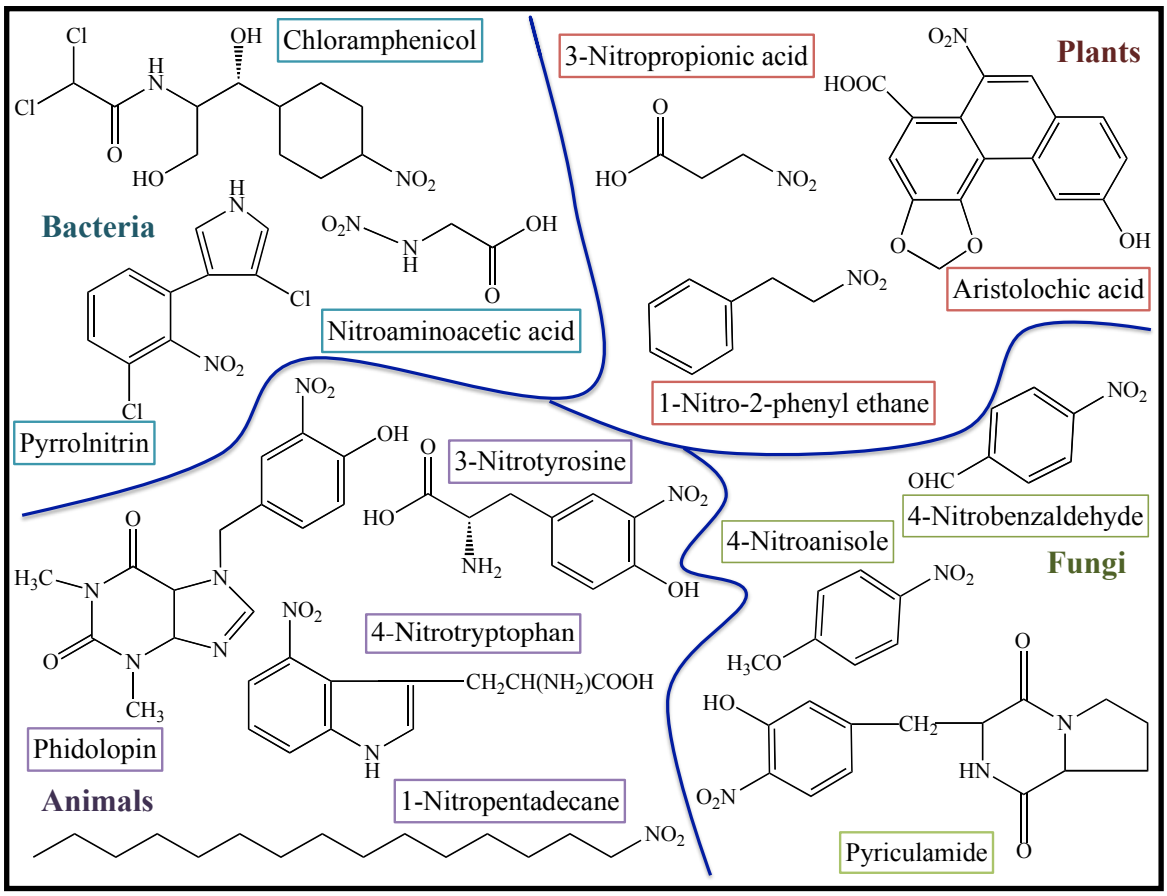

Figure 1.1. Examples of natural nitro compounds and the organisms that synthesize them.

Microbes have been shown to produce a variety of nitro compounds for use in defense mechanisms (Table 1.1). For instance, *Burkholderia cepacia*, and a variety of other strains, produce pyrrolnitrin (180), an antifungal compound, in an *N*-acylhomoserine lactone-dependent manner (144). Additionally, *Streptomyces* and *Pseudomonas* produce a variety of nitro compounds including antibiotics and phytotoxins (19, 75, 95, 144, 166). More recently, antimicrobial, as well as cyto-, phyto- and nemato-toxic, characteristics have been identified in nearly twenty different nitro compounds derived from *Salegentibacter* strain T436 isolated from Arctic sea ice (1, 145). Various fungi also produce nitro compounds that are antibiotics or toxins (168, 180). For example, pyricularin, along with other *o*-nitrophenol derivatives, isolated from *Pyricularia oryzae*, demonstrated plant growth inhibitory activities and phytotoxic effects (163). 3-Nitropropionic acid (3NPA), the first natural nitroalkyl compound described (4) is another example of a toxin produced by fungi. Chomcheon *et al* identified 3NPA in extracts of fungal endophytes including the pathogenic *Phomopsis* sp. (24). It is plausible that 3NPA is necessary for fungal infection of plants. The toxicity of 3NPA is due to its ability to function as an irreversible inactivator of succinate dehydrogenase and as an inhibitor of fumarase, succinic thiokinase and isocitrate lyase (3, 143).

Nitro compounds produced by plants commonly display various toxic effects on insects and plant pathogens (5, 6, 73, 108). For example, plant-synthesized *aci*-nitro compounds are precursors for glucosinolates, which are used as insect deterrents and fungal inhibitors (14). Recently, Bednarek *et al* discovered in *Arabidopsis* sp. the glucosinolate metabolic pathway that regulates conversion of *aci*-nitro compounds into glucosinolates to form isothiocyanates and phytoalexins for insect and fungal defense

respectively (14). Additionally, several nitrogen-fixing leguminous plants including *Astragalus, Hippocrepis* and *Indigofera* produce 3NPA (72, 73). Although 3NPA from leguminous plants may provide inorganic nitrogen in nitrogen limited ecosystems, 3NPA is implicated in poisoning a variety of livestock and insects (5) and therefore represents a source of natural nitro toxin. Both fungi and plants thus produce 3NPA, although the most substantial source of the toxin remains unclear.

Animals produce a variety of nitro compounds as either direct defense mechanisms or as a byproduct of the host immune reaction. For instance, (*E*)-1-nitropentadec-1-ene is the main chemical component of the toxins secreted by *Prorhinotermes simplex* termites for defense against predatory insects such as the house fly *Musca domestica* (99, 160, 177). The termites secrete *(E*)-1-Nitropentadec-1-ene as a chemical defense and also maintain a metabolic pathway to convert the compound into the nontoxic 1-nitropentadecane to protect termite nest mates (160). *Eucondylodesmus elegans* and other millipedes secrete 2-nitroethenylbenzenes to protect from potential predators including *Pristomyrmex pungens* ants (101). A final example is a nitroaromatic compound produced by a bryozoan. Intrigued by the absence of pathogenic microbes surrounding *Phidolopora pacifica*, Ayer *et al* identified the purine derivative phidolopin, which is an antifungal and anti-algal compound produced by the bryozoan (10).

Compounds	Organism(s) producing the compound	Ecological role of compound	Reference
4-Nitrotryptophan	*Streptomyces* sp.	Thaxtomin A intermediate	(19, 85)
Thaxtomin A	*Streptomyces* sp.	Phytotoxin	(180)
Chloramphenicol	*Streptomyces* sp.	Antibiotic	(75, 180)
Pyrrolnitrin	*Pseudomonas fluorescens*	Antifungal/antibiotic	(144)
Everninomicin	*Micromonospora carbonacea*	Antibiotic	(180)
Nitroimidazole compounds	*Streptomyces* sp.	Antibiotic/antiparasitic	(96)
4-Hydroxy-3-nitrobenzoic acid	*Salengentibacter* sp. T436	Antimicrobial/phytotoxic	(1)
4,6-Dinitroguiacol	*Salengentibacter* sp. T436	Nematicide	(1)
4,5-Dinitro-3-methoxyphenol	*Salengentibacter* sp. T436	Nematicide	(1)
2-Nitro-4-(2'-nitroethenyl)-phenol	*Salengentibacter* sp. T436	Antimicrobial/cytotoxic	(1)
3'-Nitrogenistein	*Salengentibacter* sp. T436	Cytotoxic	(1)
Phidolopin	*Phidolopora pacifica*	Antifungal/antialgal	(10)
3-Nitrotyrosine	Mammals, mollusks and plants	Unknown	(27, 69, 82, 139, 172)
3-Nitropropionic acid	Leguminous plants, fungi	Toxin, CAC cycle inhibitor	(4, 23, 24, 73)
Aristolochoic acid	*Aristolochiaceae* plants	Carcinogen, nephrotoxin	(180)

Table 1.1. Natural nitro compound examples with putative ecological roles.

There are many diverse natural analogs of each natural nitro compound, either with a similar core structure, an amino group in place of the nitro group, or simply the absence of the nitro group (179, 180). The ecological roles of such natural nitro compounds are largely unknown, however it is plausible that the compounds play several key roles including signaling and defense for the organisms that synthesize them (1, 180). There is precedent for ecological roles of antibiotic compounds and antibiotic resistance genes in the soil (26, 124), which implies that such compounds along with other natural

nitro compounds may be utilized for niche establishment and competition among microorganisms in specific ecosystem.

Although natural nitro compounds exhibit a wide array of structures, there are only two general biological mechanisms known for the formation of the nitro groups, direct nitration and enzymatic N-oxygenation (Figure 1.2) (180). In the first mechanism, direct nitration can occur via an electrophilic attack of a reactive nitrogen species, such as a nitrosyl cation on an aromatic moiety (Ar) [Ar-H $+NO_2^+ \rightarrow$ Ar-$NO_2 + H^+$] (180). The formation of the nitrosyl cation (NO_2^+) or other reactive nitrogen species including the free radical, nitric oxide ($^{\cdot}NO$), provides for an efficient attack on aromatic compounds enabling the addition of a nitro group (92, 135). An example of direct nitration is the biosynthesis of 3NTyr, whereby $^{\cdot}NO$ is oxidized to the peroxynitrite anion ($ONOO^-$) yielding nitration of protein-bound tyrosine residues (69, 82, 135). Nitration is also the key step in biosynthesis of both 4-nitrotryptophan and thaxtomin (69, 92, 94, 95, 180). Kers *et al* determined that a NO synthase (NOS) in *Streptomyces turgidiscabies* was necessary for both thaxtomin A production, presumably via nitration of tryptophan, and plant pathogenicity (92). Loss of plant pathogenicity following deletion of the NOS in *S. turgidiscabies* is intriguing (92), and logically yields the hypothesis that control of the nitration of natural compounds plays a vital function in the ecological role of such organisms.

The second mechanism by which natural nitro compounds are produced, oxidation of an amino group, is involved in the synthesis of pyrrolnitrin (71, 180) and hypothesized to be involved in chloramphenicol and 3NPA biosynthesis (23). Enzymatic N-oxygenation occurs via integration of molecular oxygen into an amino group, yielding

oxidation of the primary amine to a hydroxylamine [R-NH_2 + O_2 + 2X-H → R-NHOH +H_2O + 2X](180). N-oxygenation thus proceeds in a stepwise fashion via the hydroxylamine or subsequent nitroso species to yield the nitro moiety (13, 180). There is little information about N-oxygenases, although the enzyme required for pyrrolnitrin synthesis has been described. Hammer *et al* identified four distinct genes required by *Pseudomonas fluorescens* for the synthesis of pyrrolnitrin (63). In particular, the fourth gene that was identified was classified as a [2Fe-2S] Rieske iron dioxygenase and catalyzes the transformation of aminopyrrolnitrin to pyrrolnitrin via oxidation of the amino group into the nitro group (63, 71). For both chloramphenicol and 3NPA there is also evidence for N-oxygenase reactions in their biosynthesis. He *et al* described a putative mechanism for oxidation of the amino group of N-dicholoracetyl-p-aminophenylserinol to form chloramphenicol, but were unable to identify the specific enzyme involved (67). Candlish *et al* originally proposed the role of hydroxylamine in the synthesis of 3NPA via N-oxygenation (23). More recently, isotope labeling experiments provided evidence for the incorporation of molecular oxygen to form 3NPA, but the enzyme remains elusive (13).

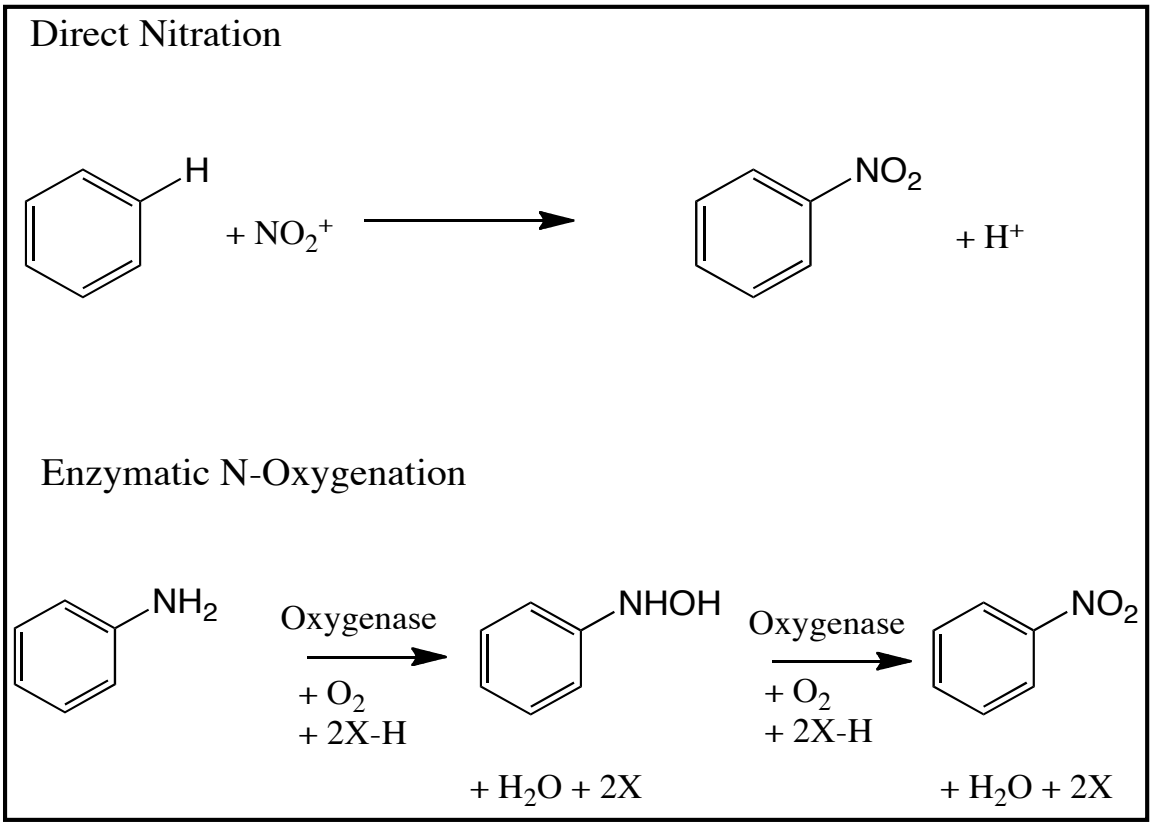

Figure 1.2. Mechanisms for addition of a nitro group to aromatic moieties (180).

1.4 Overview of anthropogenic nitro aromatic contaminants

Nitroaromatic compounds have been introduced into soil and water ecosystems following years of pesticide application and utilization as well as manufacturing of dyes, antimicrobial agents and explosives (185). TNT is a major contaminant of ecosystems (136), although many others including nitrobenzenes, mono- and dinitrotoluenes, nitrobenzoate, nitrophenols and intermediates in the synthesis and degradation of such compounds are also found in military and industrial sites (31, 38).

Synthetic nitroaromatic compounds are a concern because they negatively impact natural ecosystems and human health (55, 76, 153). Specifically, TNT can lead to liver damage and anemia in humans, and is carcinogenic in mammals (107, 112, 128). Toxicity of the intermediate compounds in the synthesis or biodegradation of TNT and other nitro compounds may be due to non-specific nitroreductase reduction of the compounds to hydroxylamine, amino, and or nitroso- derivatives, which can then hamper oxygen binding as with metahemoglobin, or be carcinogenic or bacteriostatic as in the case of nitrosoamines (55, 76).

The toxicity and intractability of nitroaromatic compounds pose a complex issue for biodegradation and biotransformation via microorganisms. It can thus be difficult to mineralize the nitro compounds to a non-toxic and potentially beneficial carbon and energy source for the microbial community. Though typically recalcitrant, many of these compounds are at least partially metabolized or immobilized by diverse microbes implying microbial adaptation to yield enzymatic pathways for synthetic nitro compound metabolism (86, 87, 97, 185).

1.5 Biodegradation of synthetic nitroaromatic compounds

During the past twenty years a substantial amount of research has been conducted to identify microorganisms capable of degrading various synthetic compounds (46, 86, 87, 97, 100, 119, 123, 154, 185). Microbes can mineralize some nitro compounds and thus obtain carbon, nitrogen and energy. In some cases, nonspecific enzymes, such as

nitroreductases in *Enterobacter cloacae* (18) or mixed enrichment cultures (118) transform nitro compounds without providing a benefit to the host microbe. The extensive list of biodegradable nitroaromatic compounds (123, 137) includes the mono- and dinitrotoluenes (87, 118, 159), mono- and dinitrophenols (103, 158), 4-nitroanisole (142), mono- and dinitrobenzenes (121, 183), and pesticides such as parathion (83).

There are four metabolic general strategies for biodegradation of nitroaromatic compounds (Figure 1.3) (119, 123, 164, 185). Degradation of aromatic moieties typically proceeds through catechol, protocatechuate, gentisate or hydroquinone analogs (173). Under aerobic conditions, several mechanisms for biodegradation are observed, including mono- and dioxygenation, partial reduction (183) and hydride-Meisenheimer complex formation, by which the nitro group is removed and the aromatic ring prepared for cleavage. Products of ring fission can then be transformed into substrates of central metabolism (123). In monooxygenation reactions the nitro group is replaced with a hydroxyl group as with 4-nitrophenol (158, 187) and 4-nitroanisole (142). Alternatively, dioxygenation produces dihydroxy- intermediates via release of the nitro group as nitrite in a variety of compounds including 2,4-dinitrotoluene (159), 2,6-dinitrotoluene (118), 2-nitrotoluene (62) and nitrobenzene (122). In another strategy, partial reduction of the nitro group permits the formation of hydroxylamino- derivatives. Further rearrangement of the hydroxylamino compound results in the release of an ammonia molecule (119) as is demonstrated in the biodegradation of nitrobenzene and 4-chloronitrobenzene (183). Finally, formation of a hydride-Meisenheimer complex via hydride transferases is involved in both the transformation of TNT (176, 181) and in picric acid degradation (105, 164) enabling nitrite release. Based on the relatively recent introduction of

synthetic nitroaromatic compounds into the environment, it is possible that the relative widespread ability of microbes to degrade nitroaromatic compounds evolved through the recruitment of enzymes already present for degradation of natural nitro compounds.

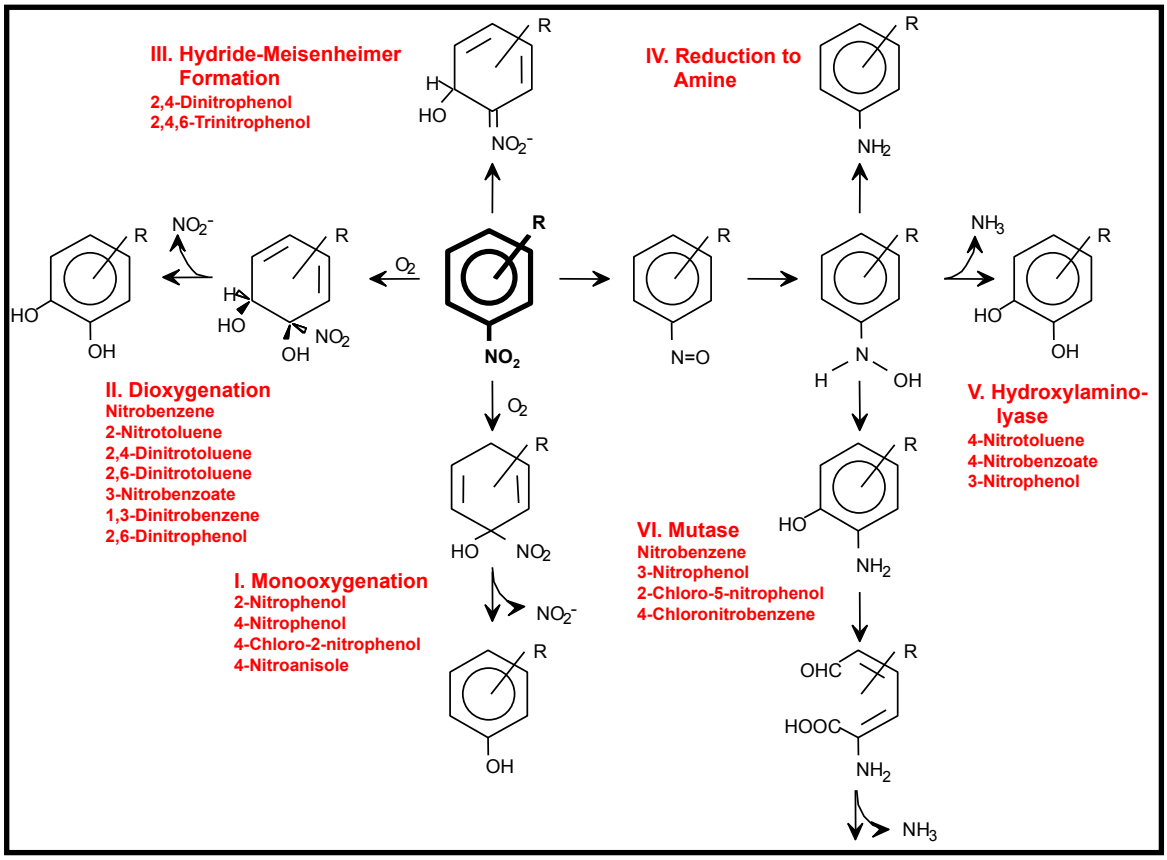

Figure 1.3. Pathways for nitroaromatic compound degradation (modified from (157)).

1.6 Biodegradation of natural nitro compounds

Several examples of the biodegradation of natural nitro compounds have been described. Schafer *et al* described two *Rhodococcus* strains capable of mineralizing 4-nitroanisole (142). The *Rhodococci* use a monooxygenase for removal of the nitro group from 4-nitrophenol, an intermediate of 4-nitroanisole degradation, and produce intermediates that are further metabolized via the citric acid (CAC) cycle (142). Evidence for the biodegradation of 3NPA and its nitronate structure has been identified in several different organisms. Anderson *et al* demonstrated that rumen microbes metabolize both 3NPA and 3-nitro-1-propanol (7). Additionally, 3NPA oxidases have been identified in vetch (72) and other leguminous plants (73). Finally, propionate-3-nitronate oxidase, a flavoprotein in *Penicillium atrovenetum* releases malonate semialdehyde, nitrite, nitrate, oxygen and hydrogen peroxide from 3NPA (25, 43, 133). Recently, the biodegradation pathway for 3NTyr in *Variovorax paradoxus* JS171 and *Burkholderia* sp. strain JS165 was biochemically characterized. Nishino and Spain demonstrated that metabolism of 3NTyr proceeds through a deamination and decarboxylation prior to the removal of the nitro group by a novel FAD monooxygenase (MO), 4-hydroxy-3-nitro-phenylacetate (HNPA) denitrase. The denitration reaction forms homoprotocatechuate, which then enters into central metabolism yielding mineralization of 3NTyr (120). The genes that encode the HNPA denitrase remain unknown.

1.7 Evolution of metabolic pathways for biodegradation of nitro compounds

The evolution of microbial metabolic pathways yields an ever-expanding assortment of potential mechanisms for biodegradation including enzyme or pathway recruitment and gene manipulation. Microbial exposure to both naturally produced and anthropogenic chemicals may enable the enzymes for natural chemical biodegradation to be recruited for use with purely synthetic chemicals (179). Genetic manipulation such as gene duplications, mutations, deletions and recombination are additional examples by which metabolic pathways can evolve (47, 87). In particular, mutations subsequent to gene duplication provide the opportunity for slight variations in enzyme structure and kinetics by which enzymatic functions with novel substrates can be developed, as has been exemplified with extradiol dioxygenases (34, 47, 48). Specifically, Eltis and Bolin compared phylogenetic relationships among extradiol dioxygenases and subsequently hypothesized that two-domain dioxygenases evolved from single domain dioxygenases by means of a gene duplication event (34). Furukawa *et al* have revealed the role of mutation and genetic recombination in the evolution of biphenyl dioxygenases through comparison of nucleotide sequences, and subsequently demonstrated that *in vitro* genetic recombination techniques permit enhanced substrate range of pre-existing dioxygenases (47, 48).

Lateral gene transfer provides for sharing and shuffling of enzymes among potentially divergent microorganisms and appears to be fundamental to the rapid construction of novel metabolic pathways for atrazine (149) and 2,4-dinitrotoluene (86,

87) among others (161, 164). For atrazine degradation, several plasmid-encoded catabolic enzymes were identified, leading to hypotheses about lateral gene transfer assisting the evolution of atrazine degradation pathways (149). Additionally, there is an initiating enzyme with broad substrate specificity (TrzN) which directs atrazine degradation, thus funneling compounds through enzymatic degradation with a narrowing substrate range (149). Also, several enzymes for nitrobenzene degradation including some nitrobenzene nitroreductase and hydroxylaminobenzene mutase enzymes have been identified as plasmid-encoded genes, implying lateral gene transfer as a mechanism for development of novel metabolic pathways for nitrobenzene (87, 131). Jeon *et al* identified several mobile genetic units surrounding genes for naphthalene degradation, hypothesizing that such mobile elements assisted in the arrangement of genes for naphthalene catabolism in *Polaromonas naphthalenivorans* (84). Finally, Johnson *et al* described the enzymatic origins for 2,4- dinitrotoluene degradation, noting that the metabolic pathways were apparently assimilated by combining genes, potentially via transposable elements, from a variety of pre-established degradation pathways including those for naphthalene, chloroaromatic compounds, and amino acids (86, 87, 164). It is plausible that discovery of novel metabolic pathways will provide further insight into the evolution of microbial degradation pathways.

1.8 Overview of flavoprotein monooxygenases

Monooxygenases are well known for their ability to catalyze a diverse range of oxidative reactions while maintaining a high level of enantioselectivity. Flavoprotein monooxygenases catalyze a wide range of reactions including hydroxylations, epoxidations, Baeyer-Villiger oxidations, sulfoxidations, amine oxidations, selenide oxidations, halogenations, phosphite ester oxidations and organoboron oxidations (111, 174).

Although the chemical reactions of flavoprotein monooxygenases are varied, the mechanism by which oxidation occurs is common. The flavin cofactors of are typically reduced by NAD(P)H via hydride transfer (12). The reduced flavin intermediate then uses molecular oxygen as a substrate, yielding an unstable peroxyflavin intermediate which decays, forming hydrogen peroxide while oxidizing the flavin (12, 111, 129, 152). The ultimate result of the flavoprotein monooxygenase reaction is the incorporation of a single atom of molecular oxygen into a reactant/substrate and the reduction of the other oxygen atom to form water concurrent with recycling the oxidized flavin moiety (88, 111).

Flavoprotein monooxygenases are structurally similar to one another. Typically, the flavin is within the substrate-binding site, so that the substrate can directly contact the flavin for efficient electron transfer (152). Binding sites for the ADP of the flavin and NAD(P) moieties within the monooxygenases are known respectively as the FAD- and NAD(P)- binding sites (110). Another key structural feature of flavoprotein monooxygenases is an encapsulating domain to contain the peroxyflavin, permitting the

stabilization of the intermediate via hydrogen bonding (2). The specific reaction carried out by the monooxygenase is dependent upon the active site chemistry and configuration, implying that fold characteristics enable distinct oxidation reactions (174).

The diversity among flavoprotein monooxygenases has prompted a variety of groups to classify the enzymes. Oxygenases have been characterized by subunit size (65) or by the number of redox active sites (152). Currently, flavoprotein monooxygenases are divided into several classes, grouped by both molecular and biochemical characteristics (111, 174). Outside of such classes, a recent review by Palfey and McDonald group flavoprotein monooxygenases into the categories 'bold' and 'cautious' based on the strategy used by the enzyme to regulated NAD(P)H oxidase activity (129).

Microbial Class A flavoprotein monooxygenases are a group of well-characterized enzymes that demonstrate high regioselectivity in diverse catalytic reactions, while maintaining structural similarity as compared to each other (88, 174). Class A enzymes typically display a narrow substrate range, catalyze aromatic compound degradation via *ortho*- or *para*- hydroxylation of the ring moiety, and use NAD(P)H as a coenzyme (174). Structurally, class A monooxygenases are encoded by a single gene and are composed of one Rossmann fold dinucleotide binding domain to tightly bind the FAD cofactor. There is no distinct NAD(P)H binding domain in class A enzymes, which is consistent with the observation that there is only a transient association with NAD(P)H during reduction of the flavin moiety (174).

There are three characteristic sequence motifs of class A flavoprotein monooxygenases (174). $GxGxxGx_{17}E$ (a βαβ FAD binding sequence motif [Rossmann fold] located near the N-terminus is involved with binding the ADP of FAD,)

FxxGDAAHxxxPxxGxGxNxsxxDsxxL (a secondary FAD-binding sequence motif, important for binding the ribose moiety of FAD) and DGxcSxhR (a NAD[P]H/FAD dual binding sequence motif that interacts with the pyrophosphate moieties of both species) (where x represents any amino acid, subscripted numbers represent the number of amino acid residues, s represents any small amino acid, c represents a charged residue and h represents a hydrophobic residue) (37, 111, 126, 138, 174). A secondary GxGxxA sequence motif is indicative of a conserved NAD(P)H binding motif (138) within a FAD binding domain.

Several class A prototype enzymes have been well characterized. The 4-hydroxybenzoate 3-monooxygenase is the most studied of the class A enzymes and catalyzes the incorporation of a hydroxyl group into *p*-hydroxybenzoate (12, 174). Two class A enzymes have been structurally characterized, a 4-hydroxybenzoate 3-monooxygenase representative from several organisms, including *Comamonas testosteroni* (74, 174) and phenol-2-monooxgenase from *Trichosporon cutaneum* (35) . The sequence and structure show that both enzymes are NADPH dependent and contain all three Class A sequence fingerprints. Interestingly, the *C. testosteroni* hydroxybenzoate hydroxylase contains a tunnel-like structural domain that apparently serves as a conduit for oxygen transport during catalysis (74). The phenol hydroxylase that Enroth *et al* described contains three distinct domains, two of which are highly similar to such domains in hydroxybenzoate hydroxlases (35). The solved crystal structures both demonstrated that class A monooxygenases share similar overall folds despite divergent sequences (35, 74).

Although class A enzymes are most relevant to this research, the other classes of flavoprotein monooxygenases are interesting based on their structural and functional comparisons to class A monooxygenases. Class B flavoprotein monooxygenases are similar to class A enzymes in that they are encoded by a single gene and contain a tightly bound FAD cofactor, however class B enzymes are functionally divergent from class A enzymes and are considered multifunctional due to their ability to oxidize both carbon- and other hetero- atoms (174). Class B flavoprotein monooxygenases are involved with a variety of catalytic reactions including biosynthesis, hydroxylation of long-chain amines, monooxygenation of diverse carbon-bound reactive heteroatoms, and Baeyer-Villiger oxidation reactions. Class B enzymes, including phenylacetone monooxygenase (109), differ from class A enzymes in that they use only NADPH and keep the coenzyme tightly bound during catalysis (174). Classes C through F are multicomponent flavoprotein monooxygenases, in which the reductase component is involved in flavin reduction, whereas the oxygenase component utilizes molecular oxygen to oxidize the substrate (88, 174). As a group, class C enzymes contain an inner core structure consisting of a TIM-barrel fold and use FMN and NAD(P)H as coenzymes (174). Bacterial luciferases, the best studied class C enzymes, are monooxygenases that emit light subsequent to monooxygenation of a long chain aldehyde (42). Class D flavoprotein monooxygenases, such as the 4-hydroxyphenylacetate 3-monooxygenase (162), are a lesser-studied group of hydroxylases that exhibit activity on aromatic substrates, and typically use FAD and NAD(P)H as coenzymes (88, 174). There is little structural information about class D flavoprotein monooxygenases, although sequence homology implies similarity to the α-helical acyl-CoA dehydrogenase fold and the kinetic mechanism for oxygenation appears

similar to that for class A enzymes (88, 162, 174). The prototype class E flavoprotein monooxygenase is the *Pseudomonas* sp. styrene monooxygenase, although few class E enzymes have been described (88, 91, 174). There is little structural information for class E enzymes, although the sequence data imply homology with class A monooxygenases, presence of a Rossmann fold-binding domain and usage of FAD and NAD(P)H as coenzymes (91, 174). Many bacterial halogenases, including tryptophan 7-halogenase, are classified as class F flavoprotein monooxygenases based on both structure and function (88, 174). Structurally, class F enzymes possess both a Rossmann fold FAD-binding domain and an additional helical substrate binding domain (174). Tryptophan 7-halogenase, encoded by *prnA*, of *P. fluorescens* and RebH, a halogenase from *Lechevalieria aerocolonigenes* are prototypical examples of such enzymes, both are FAD-dependent, use NAD(P)H as coenzymes and regioselectively chlorinate tryptophan (30, 186).

Several monooxygenases that catalyze denitration of aromatic compounds have been described (155). For example, Kadiyala and Spain characterized a two component monooxygenase from *Bacillus sphaericus* JS905 that catalyzed two monooxygenations on *para*-nitrophenol, first converting the substrate to 4-nitrocatechol and subsequently yielding nitro group removal from the 4-nitrocatechol (89). Furthermore, Spain and Gibson revealed that the initial step in biodegradation of *para*-nitrophenol by *Moraxella* sp. involves the incorporation of molecular oxygen to release nitrite (156). Zeyer and Kocher characterized a similar, yet different nitrophenol oxygenase which released stoichiometric amounts of nitrite when converting *ortho*-nitrophenol (188). Recently, the degradation pathways of several nitrophenol compounds in *Rhodococcus imtechensis*

strain RKJ300 were described. Monooxygenation yielded stoichiometric nitrite release with both 4-nitrophenol and 2-chloro-4-nitrophenol degradation (52). Finally, a monooxygenase was purified from *Burkholderia* sp. strain DNT that catalyzed the elimination of the nitro group from 4-methyl-5-nitrocatechol to form 2-hydroxy-5-methylquinone during biodegradation of 2,4-dinitrotoluene (61).

Identification and characterization of the genes that encode nitrophenol monooxygenases that catalyze denitration has yielded a greater understanding of the control of the biodegradation pathways for nitrophenols. Perry and Zylstra reported the cloning of a gene cluster necessary for *para*-nitrophenol degradation in *Arthrobacter* sp. strain JS443. *npdA2* and *npdA2* were identified as genes that encode the monomers of a two-component flavoprotein monooxygenase, the monooxygenase and the NADH-dependent flavin reductase, respectively (132). Together, NpdA1 and NpdA2 catalyze removal of a nitro group from 4-nitrocatechol, an intermediate of *para*-nitrophenol degradation. Additionally, Takeo *et al* described the gene cluster responsible for transformation of 4-nitrophenol to 4-nitrocatechol via oxygenation and nitrite removal (165). Specifically, the *nph* gene cluster from *Rhodococcus* sp. PN1 was cloned and expressed; it was determined that *nphA1* encodes the oxygenase component, whereas *nphA2* encodes the flavin reductase component. The *nph* gene cluster was determined to have similar gene organization to other nitrophenol monooxygenase operons (165), possibly implying that there is conservation among such operons. More recently, Zhang *et al* described *pnpA*, the *p*-nitrophenol monooxygenase in *Pseudomonas* sp. strain WBC-3. Interestingly, *pnpA* encodes a NADPH-dependent, single component flavoprotein monooxygenase that appears to be phylogenetically distant from other characterized

nitroarene monooxygenase (190). Although not yet described in the literature, further comparison of the genes encoding enzymes that catalyze nitro group removal from aromatic compounds would demonstrate conserved sequences among such genes and perhaps a coding region necessary for such catalysis.

1.9 Description of ring hydroxylating dioxygenases

Ring hydroxylating dioxygenases prepare aromatic ring compounds for cleavage and subsequent transformation or mineralization. Dihydroxlation commonly occurs prior to ring cleavage in order to activate the aromatic ring (54, 65, 102). Extradiol dioxygenases funnel substrates through a *meta*-cleavage pathway, as opposed to intradiol dioxygenases, which catalyze *ortho*-cleavage. Extradiol dioxygenases tend to be more versatile than intradiol enzymes (173).

Ring cleavage occurs in two discernable configurations. Cleavage in the *ortho*-configuration opens the ring between two hydroxylated carbon atoms, whereas *meta*-cleavage occurs adjacent to the hydroxylated carbon atoms (113, 173). *Meta*-cleavage yields a distinctive yellow color from the enolate anion, with wavelengths of maximal absorbance typically between 390 and 440 nm (148). *Meta*-ring fission products possess keto-dienol functional groups and share the common structure 2-hydroxy-6-oxohexa-2,4-dienoate (HODA), yet differ based on the C6 substituents (93).

Rieske non-heme iron dioxygenases, named in honor of J.S. Rieske's protein description from the mitochondrial cytochrome bc_1 complex, catalyze incorporation of

molecular oxygen into an aromatic ring or alkene to form enantiopure *cis*-dihydrodiols (54, 110, 178). There are more than 300 arene *cis*-dihydrodiols reported in the literature (54, 78, 178). The Rieske centers tend to have a high midpoint redox potential which may play a key role in funneling electron flow during oxidative catalysis, and are structurally coordinated by two histidine and two cysteine ligands (178).

The Rieske non-heme iron dioxygenases are multicomponent enzymes combining a catalytic iron-sulfur protein (ISP) terminal oxygenase with ferredoxin and reductase electron transport proteins (182). Typically the dioxygenase components are encoded by a single operon, although the ferredoxin and reductase do not appear to be dioxygenase-specific, and in several cases interact with several different Rieske non-heme dioxygenases (54, 178). The overall electron flow is from NADH to the flavin, through an electron transport chain consisting of ferredoxin and Rieske [2Fe-2S] iron-sulfur clusters, eventually reducing the terminal oxygenase (102, 110). Iron, a transition metal, often functions as a catalyst to aid initial biodegradative attack via oxygenases (65); the iron-binding site is crucial in determining the specific type of oxygenation reaction catalyzed by the enzyme (110).

The ISP has multiple subunits, α– (large, approximately 50 kiloDaltons [kDa]) and β– (small, approximately 20 kDa) composing the catalytic components of the terminal oxygenase (110). The α-subunit contains several domains: a Rieske-type [2Fe-2S] group with a mononuclear core, the non-heme iron oxygen activation center as well as the substrate-binding domain (48, 65, 178, 182). A conserved Rieske sequence motif near the N-terminus is accompanied by 2 conserved histidine and 2 conserved tyrosine residues, which reside closer to the middle of the protein and are involved in the iron

center binding domain (9, 110). ISP α–subunits appear to be related among all Rieske non-heme iron oxygenases, and many sets of degenerate primers have been designed to probe for ISP α–subunits in natural ecosystems (11, 54, 68). The C-terminal domain of the ISP α-subunit exhibits less similarity among group members than the N-terminus and is thus used to reveal phylogeny among Rieske non-heme iron oxygenases (68, 151). ISP β-subunits reveal phylogeny that is similar to that of the α-subunit, however the similarity among β-subunits is overall less than that of the α–subunits (9). The large, α–subunit is generally indicated to be key in determining substrate specificity, substrate recognition and substrate binding; however some reports describe aspects of the β-subunit as being contributory to substrate specificity (9, 47, 48, 65, 151).

Rieske non-heme iron dioxygenases tend to be similar in function and have several conserved sequence motifs, yet exhibit high diversity in substrate range based on changes in specific amino acid residues, such as in the ISP α–subunit (113, 182). Several residues including ones involved with coordination of the Rieske cluster and mononuclear iron core are completely conserved among Rieske non-heme iron and presumably necessary for the reactions catalyzed by such enzymes (39). $GxGxxGxxxGx_6G$, close to the N-terminus, is within the conserved FAD-binding sequence motif of the reductase component (110). A second GxGxxG consensus sequence approximately 140 residues towards the C-terminus contributes to the tight turn between an adjacent β-sheet and α-helix and is a highly conserved NAD-binding domain component (110). Experimental substitutions of the glycine for an alanine in the NAD-binding site alter coenzyme specificity, demonstrating why NADPH is not an effective replacement for NADH in dioxygenase systems (110). Ferredoxins contain key cysteine

residues associated with binding of the iron-sulfur cluster ($CxxxCxxCx_{29}C$) along with a secondary cysteine-rich motif ($CxHx_{15\text{-}17}CxxH$) (110). $CxHx_{15\text{-}17}CxxH$ is another consensus motif, which is required for the formation of the Rieske-type [2Fe-2S] cluster (110).

Rieske non-heme iron dioxygenases were previously classified based on redox centers and the number of enzymatic components (110). There are four families of Rieske non-heme iron oxygenases based upon classification of the ISP α–subunits and substrate specificity (Figure 1.4) (39, 54, 178, 182). More recently, a systematic classification system classifying Rieske non-heme iron dioxygenases based on oxygenase components, including type of iron-sulfur clusters in the reductase or ferredoxin components, and the presence of FAD or FMN has been proposed. The new classification system separates such enzymes into Types I through V (39, 102). The newer classification system is useful for organizing the dioxygenases, however the method separates enzymes with similar substrate ranges, making interpretation of the classification somewhat difficult.

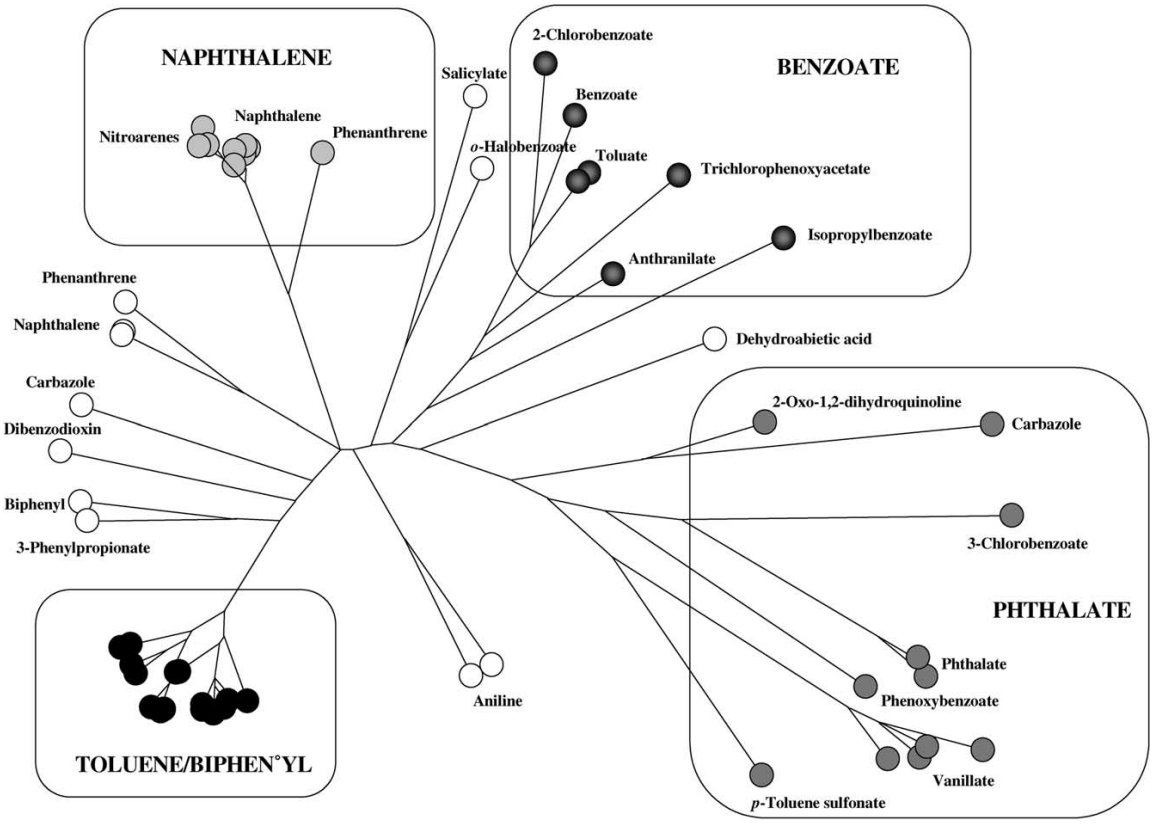

Figure 1.4. Classification of Rieske non-heme iron dioxygenases based upon ISP α-subunit (178).

Dioxygenases outside of the toluene/biphenyl family tend to be more diversified than those within the toluene/biphenyl group (54). For example, naphthalene oxygenases include those from gram-negative organisms, with enzyme specificity for both naphthalene and phenanthrene. Naphthalene dioxygenase can be further classified into Type III (naphthalene dioxygenase) and Type V (phenanthrene dioxygenase) enzymes (102). Additionally, there is one naphthalene lineage that demonstrates homology to nitrobenzene and nitrotoluene oxygenases (54). Naphthalene dioxygenases tend to oxidize a wide range of compounds, producing mostly enantiopure products. Recent

work identified a specific residue (phenylalanine 352) in the α-subunit of the naphthalene dioxygenase from *Pseudomonas* sp. NCIB 9816-4, which plays a crucial role in the production of regioselective compounds (130). Similarly, benzoate family dioxygenases (Type II) are a diverse group of enzymes that oxidize benzoate, toluate, anthranilate, 2-chlorobenzene, trichlorophenoxyacetate and isopropylbenzene (54, 102). Benzoate dioxygenases, such as the BopXYZ enzyme from *Rhodococcus* sp. strain 19070, tend to have high sequence similarity to other benzoate family dioxygenase and a broad substrate range (59). Finally, the phthalate family (Types I and V) is the most diverse group of oxygenases based upon amino acid sequence and substrate range, comprising a variety of mono- and dioxygenases with α_n-subunit configuration that oxidize aromatic acids including vanillate, phthalate, 3-chlorobenzoate, phenoxybenzoate and *p*-toluene sulfonate (54, 102). Recently, Tarasev and Ballou demonstrated that phthalate dioxygenases produce only half as much *cis*-dihydrodiol as their benzoate and naphthalene counterparts, such that only one dihydrodiol is formed for every two oxidized Rieske centers, and the mononuclear iron core remains ferrous and thus not oxidized (167). The dramatic difference between phthalate dioxygenases and other Rieske non-heme iron dioxygenases in catalysis to form dihydrodiols is remarkable. It is also of interest that the phthalate dioxygenases are phylogenetically furthest from other Rieske non-heme iron dioxygenases (Figure 1.4).

The toluene/biphenyl family of Rieske non-heme iron dioxygenases is included with the Type IV enzymes, encompasses broad substrate range enzymes and includes enzymes from both gram-positive and gram-negative organisms (102). Enzymes in the toluene/biphenyl family consist of heteromultimers of α– and β– subunits, and include

enzymes for toluene, biphenyl, isopropylbenzene, benzene and chlorobenzene oxygenation (54). The toluene/biphenyl enzymes are further subdivided into gram-positive biphenyl, gram-negative biphenyl, benzene/toluene, and isopropylbenzene families based upon the amino acid sequence of the ISP α-subunit (Figure 1.5) (182).

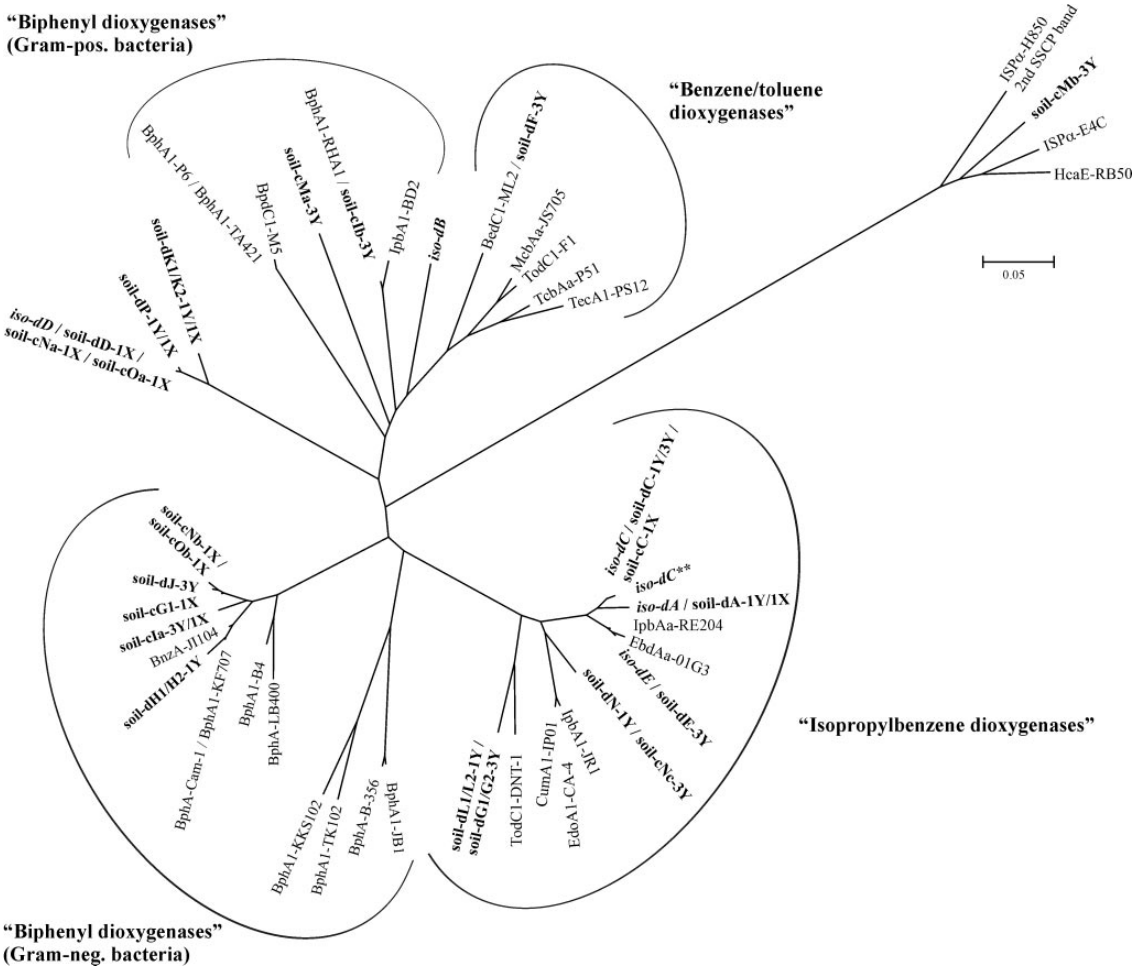

Figure 1.5. Phylogeny of ISP α-subunits demonstrating the four subdivisions of the Toluene/Biphenyl family of Rieske non-heme iron dioxygenases (182).

Toluene dioxygenases are renowned for broad substrate range (113), as well as the production of enantiopure dihydrodiols (20, 40). The well-studied *Pseudomonas putida* F1 toluene dioxygenase, encoded within the toluene operon, is derived from four genes where *todC1* and *todC2* function as a heterotetramer and encode the α– and β– subunits of the ISP respectively, *todB* encodes the ferredoxin and *todA* encodes the reductase component (150, 193, 194). In 1989, Zylstra and Gibson described the formation of *cis*-toluene dihydrodiol by *P. putida* F1 multicomponent enzyme using the pDTG601:JM109 clone, which contains the genes for the toluene dioxygenase under control of the *tac* promoter (193, 194). Since that time, the *P. putida* F1 dioxygenase construct has been widely exploited in the chemical synthesis of dihydrodiols due to its broad substrate range including synthesis of 2-methoxybiphenyl and 2,3-dimethoxybiphenyl (56), chlorobenzene (78, 79), and a wide range of brominated, chlorinated, monocyclic, fused and linked aromatics (78).

Another well-studied cluster of Rieske-type non-heme enzymes are the biphenyl dioxygenases, which represent a group of three-component enzymes. Biphenyl dioxygenases are typically composed of a large α-subunit (*bphA1*) and small β-subunit (*bphA2*) of the terminal dioxygenase as well as an electron transfer chain. The components of the terminal dioxygenase associate as an $\alpha_3\beta_3$ heterohexamer (48). Functionally, the electron transfer chain shuttles electrons from NADH to the [2Fe-2S] cluster in the α–subunit of the terminal dioxygenase via a ferredoxin (*bphA3*) and associated reductase (*bphA4)* (48). For example, the *Pseudomonas* sp. strain KKS102 biphenyl dioxygenase reductase contains both FAD and NADH binding domains, passing

electrons from the FAD to a separate [2Fe-2S] protein (ferredoxin component); the KKS102 biphenyl dioxygenase does not contain an internal [2Fe-2S] cluster (178).

There are several conserved motifs that are common to biphenyl dioxygenases and instrumental in catalysis. The $CxHx_{17}CxxH$ motif encodes the Rieske-type-[2Fe-2S] cluster, which yields the electrons that then reduce the ferredoxin (48). Biphenyl dioxygenases also contain a $GxGxxGxxxAx_6G$ putative FAD-binding site (9). Another conserved sequence, Tx_6AxGD, yields a β-sheet at the C-terminus, which is hypothesized to be required for binding the flavin via an ADP-binding site (9). Further downstream, approximately 140 amino acids from the initial glycine residue of the FAD-binding site, is the $GxGxxGxxxAx_6Gx_6E$ conserved sequence motif, which represents the ADP-binding site of the NAD-binding domain. The second glycine-rich conserved motif yields a predicted βαβ-fold that is involved with biphenyl dioxygenase catalysis (9).

As an enzyme family, biphenyl dioxygenases demonstrate well-characterized abilities to transform a wide variety of compounds into the corresponding *cis*-dihydrodiols through relaxed substrate specificity. Potential substrates for biphenyl dioxygenases include poly-chlorinated compounds (>200 structures), as well as benzene and biphenyl compounds with methyl, ethyl, vinyl, carboxyl, halogen or nitro substituents (175). Biphenyl dioxygenases are also capable of other catabolic activities with a number of aromatic compounds including dehalogenation, monooxygenations, sulfoxidations, dealkylation and desaturation reactions (175).

Some biphenyl dioxygenases exhibit a greater substrate range than others. The *Burkholderia* sp. LB400 biphenyl dioxygenase oxidizes a wider range of polychlorinated biphenyl compounds than the *Pseudomonas pseudoalcaligenes* KF707 biphenyl

dioxygenase (48, 54). Specifically, the biphenyl dioxygenase of *Burkholderia* sp. LB400 is believed to have relaxed regiospecificity such that molecular oxygen can be incorporated at the *ortho* chlorinated position yielding subsequent elimination of the chloro-substituent (8). The difference between the LB400 and KF707 biphenyl dioxygenases is a 21 amino acid change among 460 overall residues in the ISP α-subunit, with all but one of the amino acid changes occurring in a short, 140-amino acid region of the C-terminus (48, 114). There are four distinct regions of the ISP α-subunit based on sequence analysis. Changes in amino acid residues from the sequence of LB400 *bphA1* to the sequence of KF707 in Regions 1, II or IV yield no significant changes in substrate specificity (114). However, changes in the third sequence region of the ISP α-subunit of LB400 *bphA1* to the KF707 sequence yielded a significant change in substrate specificity (114), although residues outside of Region III are also acknowledged to influence the enzymatic substrate range (175). The amino acid differences between the ISP α-subunit of LB400 *bphA1* and the KF707 *bphA1* that yield great changes in substrate specificity all occur in between Regions III and IV (114). The LB400 biphenyl dioxygenase Region III sequence Thr^{335}-Phe^{336}-Asn^{337}-Asn^{338}-Ile^{339}-Arg^{340}-Ile^{341} contains four different residues from the sequence identified in *P. pseudoalcaligenes* KF707 and has a much wider substrate range with chlorobiphenyls (175). In particular, the first two residues of Region III appear to play a crucial role in substrate specificity and substrate binding as identified in experiments using site directed mutagenesis to alter the threonine-phenylalanine residues, whereas residues 338 and 341 appear to have key roles in turnover rates and catalytic activity towards substrates (114, 175). Site-directed mutagenesis experiments have pinpointed key amino acids in the biphenyl dioxygenase of *Burkholderia* sp. LB400,

yielding a variety of key residues that both contact the substrates and are hidden within protein folds (191, 192). Further studies will be necessary to determine key residues and substrate ranges of additional biphenyl dioxygenases and biphenyl dioxygenase-like enzymes.

1.10 Overview of hydrolases

Along with the biphenyl dioxygenase, the 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (involved with biodegradation of biphenyl) is another class of enzymes with a combination of structure and catalytic mechanisms to influence substrate range specificity (104, 147, 148). Hydrolases involved with biodegradation of biphenyl are members of the α/β hydrolase-fold superfamily, and tend to be substrate specific (49, 93), with multiple hydrolases in a single organism conferring an increased substrate range to the host (184). The hydrolases share a similar 3D core structure, have a varied primary sequence, and exhibit divergent catalytic capabilities with similar enzymatic mechanisms (29). Thus, structure is more important for catalytic activity than primary sequence (49). Although, several conserved sequence motifs have been identified as being characteristic of hydrolases and necessary for catalysis (29, 41, 93). The sequence differences among hydrolases are increased in the N-terminus and central regions of the hydrolases, and the central region is hypothesized to play a crucial role in determining substrate specificity (41, 93). Further study of hydrolases is necessary to reveal the impact of small sequence changes in the central region upon substrate specificity.

The conserved catalytic triad confers the ability to cleave esters, amides, carbon-halogen bonds and carbon-carbon (C-C) bonds (29); in biphenyl for instance, the C-C bond cleavage yields 2-hydroxypenta-2,4-dienoate and benzoate (148). The subsequently cleaved C-C bond lies between a dienoate (C1-C5) and a carbonyl (C6) (146). Hydrolases convert specific substrates via enol-keto tautomerization prior to hydrolysis such that initial nucleophilic attack by a catalytic serine on the ketonized substrate generates a *gem*-diol intermediate, followed by generation of an acyl-enzyme intermediate and subsequent product release. Activation of water by means of a catalytic triad histidine yields release of the final product and recycling of the hydrolase (77, 93).

The catalytic triad in hydrolases contains a nucleophile-acid-histidine motif where the nucleophile is typically a serine residue and aspartic acid is typically the acid residue (29, 41, 93, 104). The nucleophile, acid residue and histidine are separated by a number of residues, which vary by enzyme, yet the catalytic triad folds into similar topological configurations within the functional enzyme among hydrolases (29, 41, 93). As such, the nucleophilic residue is within a 'nucleophilic elbow' motif, contained in the consensus sequence SmxNuxSmSm, where Sm represents a small amino acid, i.e. glycine, x represents any amino acid and Nu is the nucleophile, typically serine (29, 93). Changes in the 'nucleophilic elbow' motif can yield a significant impact on enzymatic activity. For example, hydrolases capable of degrading monocyclic compounds have a phenylalanine after the nucleophilic residue, whereas hydrolases involved in catalysis of bicyclic compounds tend to have a methionine residue following the nucleophile (93). Residues surrounding the nucleophile, but outside of the elbow motif, tend to not be conserved and thus are presumed to have little impact on catalytic functions (29).

There are several reported crystal structures that yield insight into the catalytic mechanism of hydrolases involved in the degradation of biphenyl (BphD from *Rhodococcus* sp. strain RHA1 and *Burkholderia* sp. LB400 (77, 106, 115)), cumene (CumD from *Pseudomonas fluorescens* IP01,(49)), carbazole ($CarC_{J3}$ from *Janthinobacterium* sp. strain J3,(58)) and phenylpropionate (MhpC from *Escherichia coli*,(106)). The hydrolases all consist of a similar structure, which is an eight-stranded β-sheet surrounded by a core domain of α-helices, with a lid domain inserted within the core (29, 93, 146)**.** Movement of the α-helices within the lid domain greatly alter the shape of the substrate binding pocket (49). The crystal structures demonstrate that the active catalytic residues sit within the core domain, facing towards the lid domain (49, 77), and the active site with the catalytic triad sits at the cleft between the core and the lid domains (77). In particular, Li *et al* identified the key residue arginine 188, which is conserved among all α/β hydrolase enzymes, as being necessary for hydrolytic cleavage in both BphD and MhpC (106).

Members of the α/β hydrolase-fold superfamily were initially characterized based upon primary sequence, however further analysis has led to the classification of such enzymes into four families based upon catalytic abilities (Figure 1.6) (93). Specifically, families I and III include hydrolases that degrade both monocyclic and bicyclic compounds, whereas family II hydrolases are known for their ability to degrade carbazole, fluorene, naphthalene and phenylpropionic acid, along with related substrate derivatives. Finally, family IV hydrolases degrade monocyclic compounds (93).

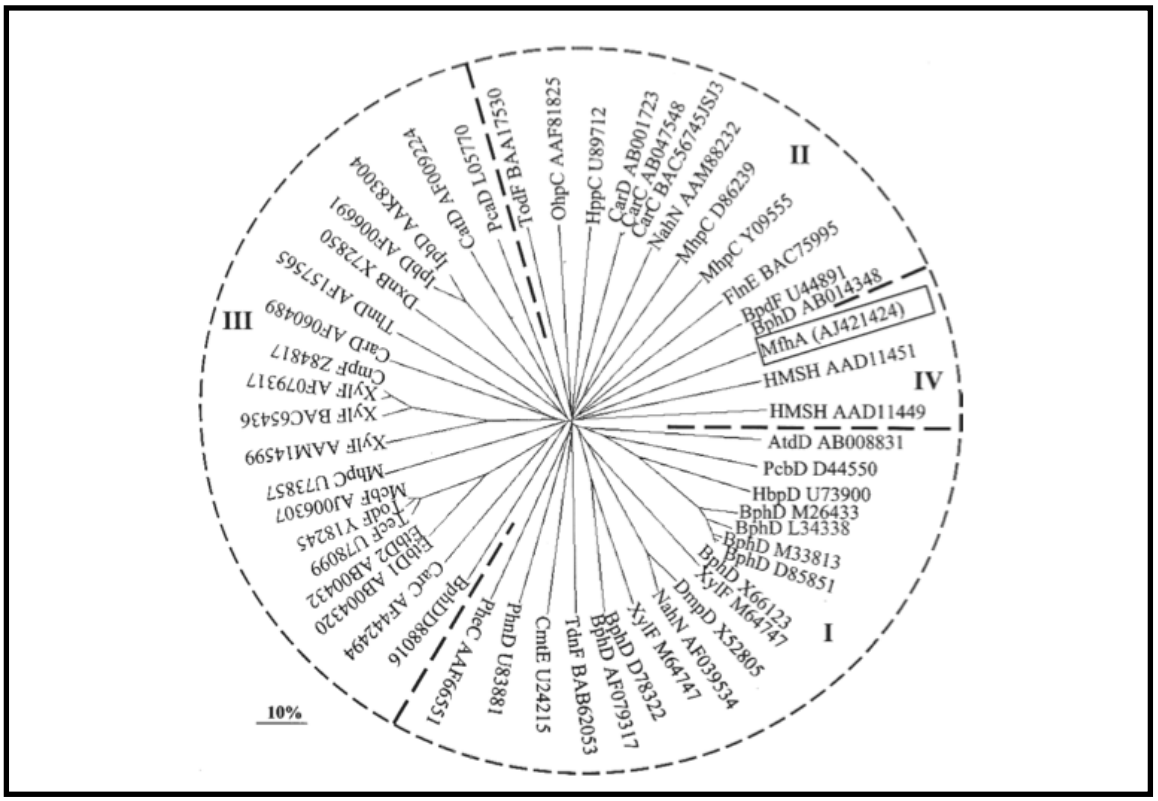

Figure 1.6. Phylogeny of *meta*-ring fission product hydrolases. Scale bar represents the percentage of divergence (93).

1.11 Potential manipulation of enzymes for green chemistries and increased substrate range

Engineering naturally occurring oxygenases to augment catabolic activities with anthropogenic contaminants is of great potential for green chemistries for biocatalysis. In particular, production of enantiopure dihydrodiols is of interest as such compounds are useful precursors for biosynthesis of indigo (36), prodrugs (80) and as precursors for synthesis of other compounds including morphine intermediates (20, 78). For example,

toluene and biphenyl dioxygenases have been manipulated via site directed mutagenesis to improve substrate range (22, 47, 125). The close sequence similarity between the ISP α-subunits of toluene and biphenyl dioxygenases has enabled several hybrid enzymes to be engineered, combining toluene and biphenyl dioxygenase subunits, as well as multiple biphenyl dioxygenase subunits from different organisms, yielding enzymes with increased and divergent substrate ranges (9, 22, 47, 125). A hybrid enzyme of *Pseudomonas pseudoalcaligenes* F1 *todC1* and *bphA2A3A4* from *P. pseudoalcaligenes* KF707 converts toluene, benzene, styrene, *p*-xylene, acetophenone, propiophenone, 2,2,2-trifluoroacetophenone and butyrophenone to the corresponding *cis*-dihydrodiols (150). Camara *et al* developed another hybrid dioxygenase expression system, based on a twenty-four amino acid residue difference, using either *bphA1* of *Burkholderia xenovorans* LB400 or *Pseudomonas* sp. strain B4-Magdeburg along with *bphA2A3A4* of *B. xenovorans* LB400 (22). Such hybrid dioxygenases demonstrated greatly increased substrate ranges and conversion efficiencies with *ortho-, meta-,* and *para*-chlorinated ring compounds and showed increased regiospecificity, thus yielding novel dioxygenase activities via genetic modification (22). The diverse substrate specificity of toluene and biphenyl enzymes in natural ecosystems remain a mystery, but more information would greatly enhance the construction of hybrid dioxygenase expression systems for increased substrate range.

1.12 Importance of functional annotation

The tremendous increase in affordable sequencing has yielded a plethora of genome sequencing projects, providing ample sequence information; a drawback to such research endeavors is the difficulty of correct annotation. Draft sequences are often published as cheaper and faster alternatives to finished genomes (141). Such incomplete sequences often contain erroneously assembled and annotated contigs, which are often deposited in databases such as GenBank (http://www.ncbi.nlm.nih.gov/GenBank/index.html). As GenBank entries are only able to be corrected or edited by the submitting author, several un-annotated and misannotated entries remain in the database, perpetuating error every time such entries are used for comparison to newer entries (141). Thus, there is a tremendous number of unknown open reading frames (ORFs), coupled with a great amount of incorrectly annotated ORFs in current genomic databases (28, 51, 141). Unknown ORFs are common even in well-studied genomes including *Escherichia coli* and *Bacillus subtilis,* in which approximately 40% of ORFs cannot be annotated (51, 117). Assigning gene function based on sequence similarity is a common practice, however the use of the closest homolog for gene annotation is unfortunate in that sequence similarity among homologs is not entirely sufficient for adequate annotation (16, 51, 117). The hypothesis that a given homolog maintains similar gene function is plausible, but requires biochemical evidence for proof or correction of the annotation (127). Based on the number of major enzyme superfamilies, it is likely that the majority of structural analogs must encode enzymes with different functions (16, 117).

Errors in gene annotation are common and possible even when similarity is high (greater than 80%) (51, 117). Functions of enzymes are potentially assigned incorrectly in greater than one third of annotations (28). Additionally, disparities among different groups annotating the same genome may be great, even when using similar data and methods. For example, in 1999, Brenner identified an error rate of greater than 8% among several groups annotating *Mycoplasma genitalium* when comparing differences of annotation by one group as compared to the other two performing annotation (16). The error rate for *M. genitalium* annotation is presumably much higher if error rates among all three annotating groups were compared separately and equally (16, 28).

Once gene annotation has been conducted, either *in silico* or manually, the gene function information is entered into public databases. Such practice propogates annotation errors by becoming the basis for subsequent annotation of further genomes based on homology (16, 28, 51, 141). Therefore, there is a widening gap between generated sequence data and the biochemically characterized enzymes studied in labs (117).

Lessening the gap between sequence data and functionally characterized enzymes is necessary to improve understanding of the proteome of individual organisms as well as functional proteomes in ecosystems. Thus re-annotation of genomes based on biochemically characterized gene products is becoming a necessary trend in research (117, 127). Discovery of novel classes of enzymes and individual gene functions will reveal metabolic diversity and aid in annotation and annotation correction for further gene discovery and characterization.

1.13 3-Nitrotyrosine

3NTyr is a post-translational modification that is a biological marker for inflammation in mollusks (172), plants (139) and mammalian diseases including cystic fibrosis, asthma and diabetes (15, 33, 69, 81, 82). Specifically, nitration of protein-bound tyrosine, is related to the biosynthesis of nitric oxide (NO) (82, 135, 139, 172, 180). A range of organisms, including microorganisms generate nitric oxide (NO); which is often produced during metabolism or infection response and is used both as a non-specific host defense mechanism and as an important cell signaling molecule (81, 82). Mollusks and mammals produce NO as part of a host inflammatory response involving NO synthase (NOS) (21, 33, 50, 69, 81, 171). Plants such as soybean and tobacco produce also both NO and 3NTyr, however the mechanism by which NO production yields 3NTyr is significantly different from that in mammals and mollusks because the ratio of NO to reactive oxygen intermediates determines the production of peroxynitrite ($ONOO^-$) in plants (27, 139).

Several mechanisms have been described that yield tyrosine nitration and subsequent disruption of biological processes involving nitrated proteins. Specifically, biological and chemical transformations of NO yield a cascade of reactive nitrogen species, which can modify a variety of macromolecules, including protein-bound amino acids. For instance, in the presence of the superoxide anion, NO yields $ONOO^-$, which nitrates tyrosine residues in proteins (32, 82, 135). Such nitration of amino acids results in the disruption of cell signaling, as well as variation of the overall protein function via conformational changes or restrictions on protein activation (135). Another described

method for tyrosine nitration involves atmospheric nitrous acid (HNO_2), which also nitrates proteins, including tyrosine residues in pollen (44). It is plausible that 3NTyr is released when nitrated proteins are degraded, however both the impact and fate of 3NTyr is unknown. Although there is substantial research on 3NTyr, the research focus has primarily been on the physiological cause and analysis of the modification in disease states (32, 33, 50, 69, 81, 82, 172), leaving the biological effect of tyrosine nitration a mystery.

The degradation pathway for 3NTyr is known for two isolated bacteria, *Variovorax paradoxus* JS171 and *Burkholderia* sp. JS165 (120), however its distribution, metabolic variability and significance in ecosystems have yet to be elucidated. The isolates use 3NTyr as the sole source of carbon, nitrogen and energy through a distinct biochemical pathway (Figure 1.7). The key enzyme is HNPA denitrase, and the activity of the enzyme can be monitored via the release of nitrite during 3NTyr degradation (120). Although the catabolic steps required for 3NTyr biodegradation have been elucidated, the enzymes and genes that encode them have yet to be described.

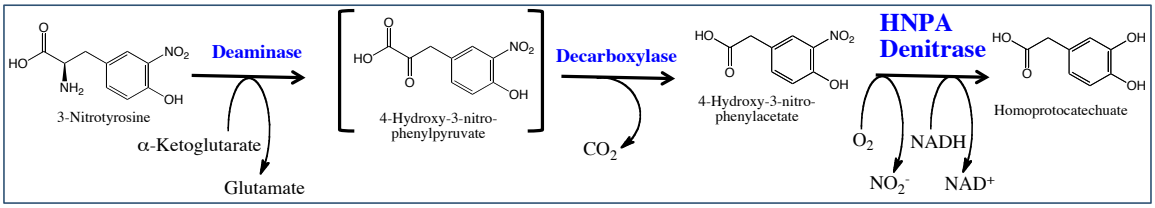

Figure 1.7. Degradation pathway for 3NTyr in *Variovorax paradoxus* JS171 and *Burkholderia* sp. JS165, modified from (120).

1.14 1-Nitro-2-phenylethane

NPE is a nitroaliphatic ring compound produced by a variety of plants including tomato plants (4, 169), *Tropaeolum majus* (4), Wright's gardenias (90), *Ocotea pretiosa* (4, 57), *Aniba canelila* (57, 116), jasmine (134) and orchids (90); however, the ecological significance and metabolism of NPE remain a mystery. The lack of accumulation of NPE in ecosystems implies degradation or turnover of the compound.

Other than its characterization as a volatile organic compound in a variety of plants, little is understood about the biological function of NPE; however comparisons with other secondary plant metabolites provide possible ecological roles for NPE. NPE is derived from the shikimate pathway, originating with phenylalanine and proceeding through an aromatic amino acid decarboxylase pathway (70, 98). Tieman *et al* suggest NPE biosynthesis occurs via oxidation of an amine group (169). Specifically, NPE is formed from a phenylalanine precursor, along with other aromatic and aliphatic compounds including 2-phenylacetaldehyde and 2-phenylethanol, (Figure 1.8) which appear to function either as a flavor or scent to attract insects for pollination, or as a pesticide-related compound (98, 169, 170). For example, 2-phenylethanol has antimicrobial properties and together with 2-phenylacetaldehyde, the chemicals function as attractants for predatory insects (4, 98, 169, 170). NPE is also presumed to be intermediate in glucosinolate biosynthesis; glucosinolates appear to function as protection against plant predators (4, 64, 108). There are multiple potential roles for NPE in both plant defenses and pollination/reproduction (4, 169). It is thus possible that NPE serves to repel insect or plant predators (based on structural and pathway similarities), or as a

chemical attractant as do other similarly produced compounds, i.e. 2-phenylethanol and other glucosinolates (4, 64).

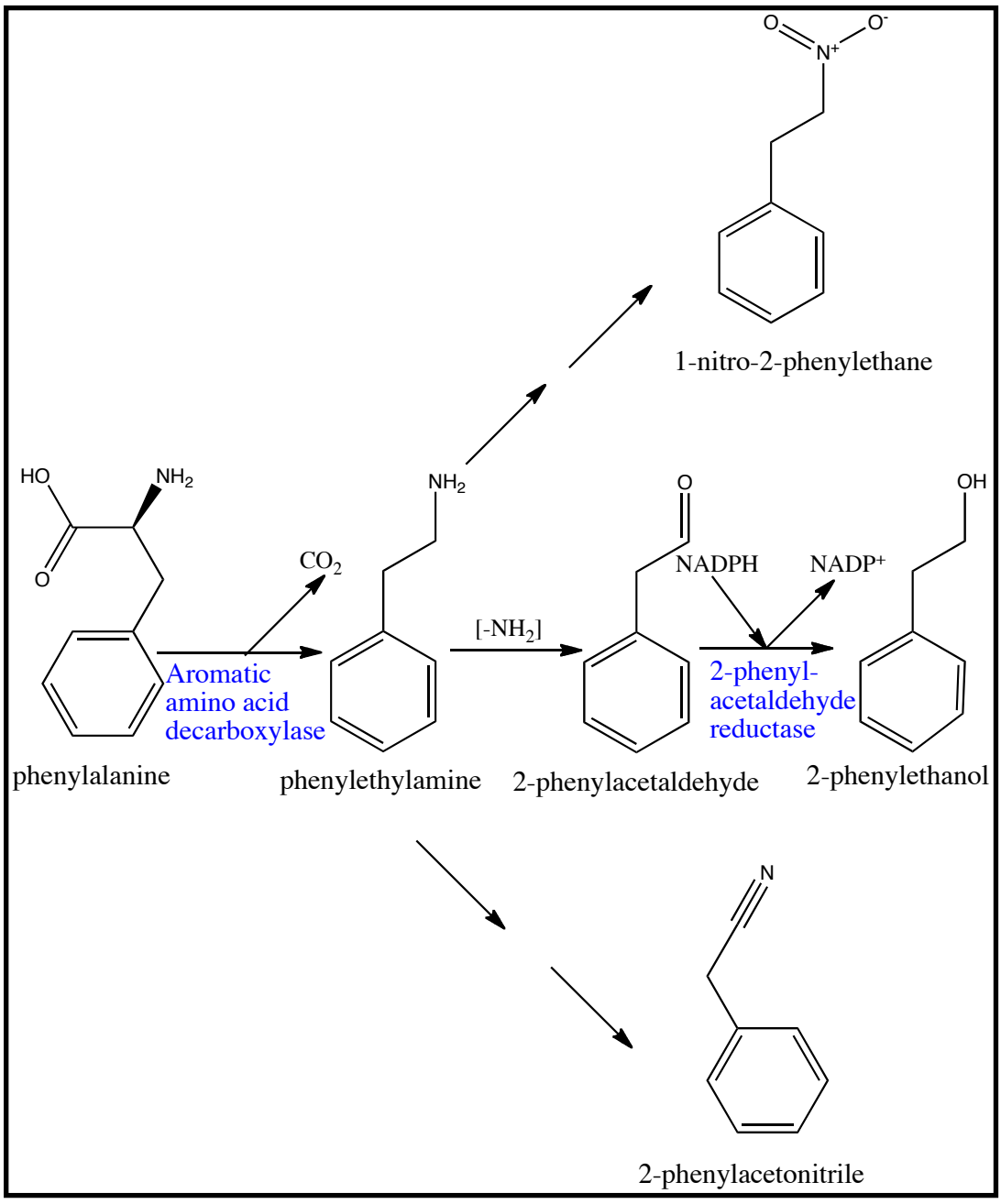

Figure 1.8. Formation of NPE concurrent with phenylalanine metabolism and synthesis of 2-phenylethanol and 2-phenylacetonitrile (modified from (169)).

1.15 Potential scientific contributions from this thesis research

There is a lack of knowledge as to the ecological purpose and biological impact of catabolism of natural nitro compounds. Additionally, nothing is known about the ecology, flux or degradation of nitroaliphatic compounds. As described above, characterization of the microbial catabolic pathways that have evolved to degrade natural nitro compounds will yield further understanding about microbial degradation of synthetic nitro compounds. Description of biodegradation pathways, key enzymes and the genes that encode them for the natural nitro compounds 3NTyr and NPE will identify novel microbial metabolism, yield insight into the ecological impact of natural nitro compounds, improve gene annotation and reveal novel enzymes for future green chemistry applications.

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CHAPTER 2

DISCOVERY OF THE GENE THAT ENCODES 4-HYDROXY-3-NITRO-PHENYLACETATE DENITRASE, THE KEY ENZYMATIC STEP IN 3-NITROTYROSINE DEGRADATION IN *VARIOVORAX* SP. JS669

2.1 ABSTRACT

3-Nitrotyrosine (3NTyr) is produced by a common post-translational protein modification at the locations of infection and inflammation in plants and animals. The biodegradation pathway for 3NTyr was recently described, however the gene for the key denitrase enzyme has not been reported. We identified the gene (*denA*) that encodes the key enzyme, 4-hydroxy-3-nitro-phenylacetate (HNPA) denitrase, which we have designated as DenA in *Variovorax* sp. JS669, using a combination of molecular, biochemical and bioinformatics methods. *denA* was significantly upregulated in cells grown on 3NTyr. A *denA* mutant failed to degrade HNPA but remained capable of slow growth on 3NTyr. DenA is an inducible enzyme that represents a novel clade of FAD monooxygenases. The identification of *denA* establishes the likely function of several genes in the same clade whose roles were previously unknown. The insight about the molecular biology of *denA* enables further study of the HNPA denitration mechanism and the ecological and biological impact of 3NTyr metabolism.

2.2 INTRODUCTION

Natural nitro compounds are produced by all domains of life and represent a tremendous amount of diversity in structure and function (3, 10, 17, 35, 46, 47, 49). Yet for the vast majority of natural nitro compounds, nothing is known about their metabolic and ecological significance. That natural nitro compounds seem to not persist in natural ecosystems implies that there is a flux of the compounds among organisms that produce the nitro compounds and organisms that are capable of biodegrading them.

3-Nitrotyrosine (3NTyr) (a natural nitroaromatic compound) is a post-translationally modified, protein-bound tyrosine, associated with infection and inflammation in a wide variety of organisms (8, 12, 15, 17, 46). There are over 3000 references specific to 3-nitrotyrosine (3NTyr) in the scientific literature (Scifinder [American Chemical Society] March 2010). In addition there are numerous naturally occurring analogs of 3NTyr including pyriculamide, phidolopin, and 2-nitro-4-(2-nitroethenyl)phenol (1, 3, 37). Denitration of 4-hydroxy-3-nitro-phenylacetate (HNPA) is the key step in the degradation pathway for 3NTyr (Figure 2.1) (27), as well as the catabolic pathway for 3-nitrotyramine (6). Despite reports of metabolism of both 3NTyr (27) and 3-nitrotyramine (31) in microorganisms, the gene encoding the HNPA denitrase has not been previously identified.

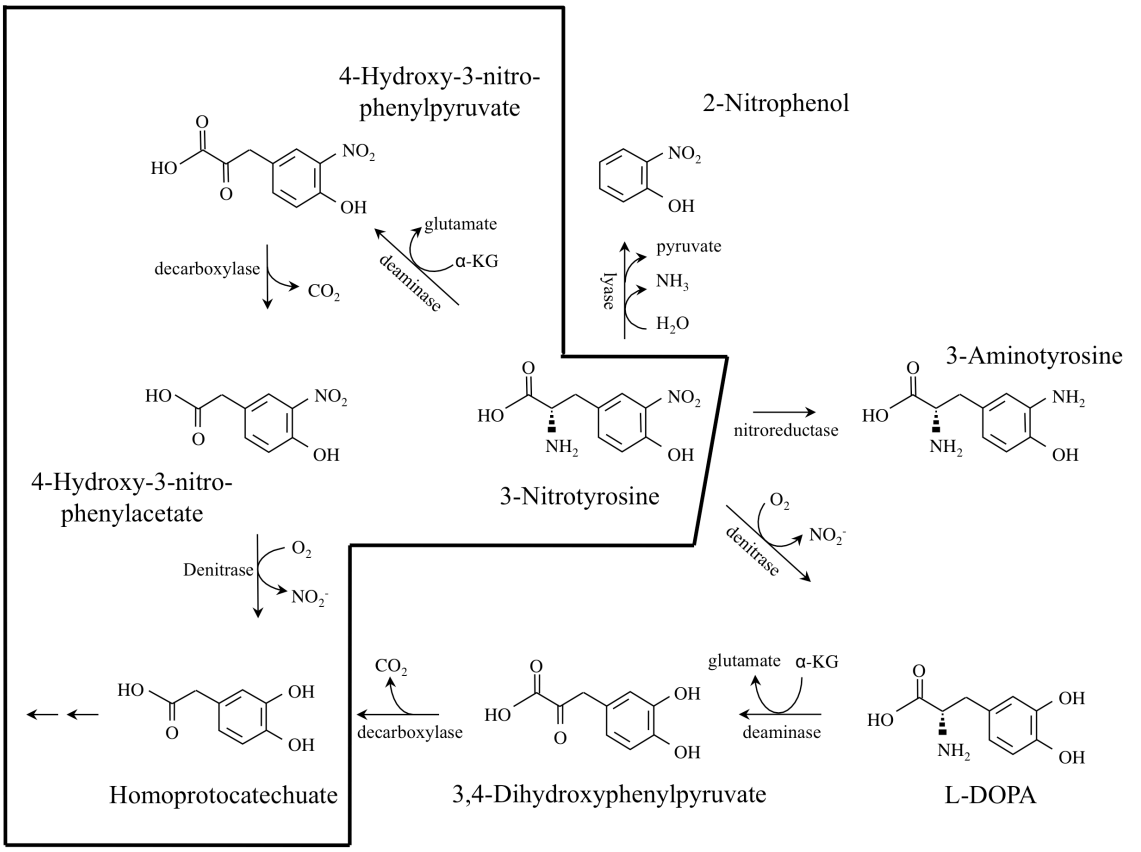


Figure 2.1. Potential pathways for 3NTyr metabolism. The outlined pathway is the reported 3NTyr degradation pathway in *Variovorax paradoxus* JS171 and *Burkholderia* sp. JS165 (27).

Selective enrichment from soil using 3NTyr as the carbon and nitrogen source yielded *Variovorax* sp. JS669, which used the previously described 3NTyr degradation pathway (27). We report here the identification and characterization of *denA*.

2.3 MATERIALS AND METHODS

2.3.1 Isolation and growth of bacteria. Bacteria able to grow on 3NTyr were isolated from soil (Tifton, GA) by selective enrichment with 3NTyr as the sole carbon and nitrogen source (27). Cultures were maintained on BLK (7) plates (1.5% agar) supplemented with 3NTyr (500 μM). Liquid cultures were grown in BLK containing 3NTyr (500 μM) both with and without glucose (5 mM) as an additional carbon source. *Bradyrhizobium japonicum* USDA110 and *Bradyrhizobium* sp. BTAi were assayed by auxanography for their ability to grow using 3NTyr and/or HNPA as the sole carbon, nitrogen and energy source (29). *E. coli* was routinely grown at 37^{o}C on Luria-Bertani Broth (LB) (36). When required for induction, isopropyl β-D-1-thiogalactopyranoside (200 μg/ml) (IPTG) was included. A complete list of strains and plasmid used in this study is presented in Table 2.1.

Strain/Plasmid	Genotype/Description	Reference
Strains		
E.coli S17-1	RK2 *tra* regulon, *pir*, host for *pir*-dependent plasmids	(23)
Variovorax sp. JS669	3NTyr-degrading isolate	This study
Variovorax paradoxus JS171	3NTyr-degrading isolate	(29)
JS669*denA*::pVIK110	Kan^r, insertional mutant in *Variovorax* sp. JS669 *denA*	This study
Bradyrhizobium japonicum USDA 110	Strain with putative FAD MO with high similarity to DenA	(10)
Bradyrhizobium sp. BTAi	Strain with putative FAD MO with high similarity to DenA	(9)
Plasmids		
pVIK110	Kan^r, *pir*-dependent suicide vector	(23)
pJS337	Amp^r, *denA* from *Variovorax paradoxus* JS171 cloned into pBAD	(30)
pJS338	Amp^r, putative *denA* from *Bordetella bronchiseptica* RB50 cloned into pBAD	(30)
denA:pVIK110	Kan^r, partial fragment of *denA* from *Variovorax* sp. JS669 cloned into pVIK110	This study

Table 2.1. Bacterial strains and plasmids used in this study.

2.3.2 Respirometry. Induced cultures were grown in BLK supplemented with glucose (5 mM) and 3NTyr (500 μM); control cultures were grown in BLK medium with glucose (5 mM) and NH_4Cl (1 mM). Cells were harvested in exponential phase by centrifugation (10,000 x g, 4°C, 20 min), washed twice with phosphate buffer (20 mM; pH 7.0), and suspended in air-saturated phosphate buffer. Oxygen uptake was measured polarographically at 25°C with a Clark-type oxygen electrode connected to a YSI model 5300 biological oxygen monitor.

2.3.3 Cell extracts. Washed cells were passed three times through a French pressure cell (20,000 psi). The cell extract was centrifuged (20,000 x g, 4°C, 20 min) to remove the cell debris.

2.3.4 Analytical methods. Nitrite concentrations were measured colorimetrically as previously described (25). Protein was measured using a Pierce BCA protein assay reagent kit (Rockford, IL). HPLC analyses were conducted as previously described (27).

2.3.5 DNA extraction and plasmid isolation. DNA was extracted from individual colonies using the SV Wizard Genomic DNA Purification kit (Promega, Madison, WI). Plasmid DNA was isolated using the Wizard SV Miniprep kit (Promega, Madison, WI).

2.3.6 PCR conditions, primer design and DNA sequencing. PCR amplification of the partial 16S gene from isolates was conducted using primers 341F and 926R (4) (Table 2.2). PCR reactions with other primer sets were similar to the 16S reactions except for changes as described (Table 2.2). Amplicons were purified using Wizard SV Gel and PCR Clean up Systems (Promega, Madison, WI) and sent to the Nevada Genomics Center (Reno, NV) for sequencing. Sequences were reviewed and compared with sequences in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/index.html) via BLASTN analysis (2). Primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Primer	Sequence	Ta[1] (°C)	Time[a] (sec)	Time[e] (sec)	[$MgCl_2$] (mM)	Source
DenGenF	5'- AAYGTNAARTGYAAYCAYGT	43	30	60	7.5	(30)
DenGenR4	5'- GCRTTCATNCCRTANCCNGC	43	30	60	7.5	(30)
CEKG2A	5'- GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN AGA G	TD[2]	30	180	NA[3]	(18)
CEKG2B	5'- GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN ACG CC	TD[2]	30	180	NA[3]	(18)
CEKG2C	5'- GGC CAC GCG TCG ACT AGT ACN NNN NNN NNN GAT AT	TD[2]	30	180	NA[3]	(18)
CEKG4	5'- GGC CAC GCG TCG ACT AGT AC	50	30	180	NA[3]	(18)
341F	5'- CCTACGGGIGGCIGCA	50	30	60	3.125	(4)
926R	5'- CCGICIATTIITTTIAGTTT	50	30	60	3.125	(4)
KOdenAF	5'- AATCGAACGCGACTCACATC	64.2	60	60	2.5	This study
KOdenAR	5'- TGGTGCTACTACTCGGTCAA	64.2	60	60	2.5	This study
rpoB 1698F	5'- AACATCGGTTTGATCAAC	50	90	90	2.6	(9)
rpoB 2041R	5'- CGTTGCATGTTGGTACCCAT	50	90	90	2.6	(9)
denApet21F	5'- CCCGCCCGCATATGGAAAACAT CGACACGGACG	64	60	90	1.5	This study
denApet21R1	5'- CCGAAGCTTGATCCATGGCCCC GGATTCAGG	64	60	90	1.5	This study
502F	5'- AGCGAGACCTTTCGGCATATTC GT	56.8	60	60	2.5	This study
LacZR	5'-CGCCAAGACTGTTACCCATC	54.4	60	120	2.5	This study

[1] Annealing Temperature, [a] Annealing Time, [e] Extension Time, [2] details in Jacobs *et al* 2003, [3] Phusion Taq Master Mix containing $MgCl_2$ (New England Biolabs, Ipswich, MA).

Table 2.2. Primers and PCR conditions. PCR reactions contained template DNA along with 400 μM dNTPs, 1 μM combined forward and reverse primers, $MgCl_2$, 1X GoTaq Flexi Buffer and 1u Go Taq DNA Polymerase (Promega, Madison, WI). All PCR conditions began with an initial denaturation (95°C, 5 min) and continued with 35 cycles of denaturation (95°C, 2 min), primer annealing, and DNA extension. PCR cycles concluded with a final extension (72°C, 10 min).

2.3.7 RNA extraction and reverse transcription. Cultures of *Variovorax* sp. JS669 were grown in 100 ml of nitrogen-free BLK containing glucose (5 mM) and either 3NTyr (500 μM) or NH_4Cl (9.4 mM) as the nitrogen source. Exponential phase cells were harvested by centrifugation, and the supernatant was monitored for 3NTyr disappearance by HPLC. Cell pellets were incubated in 0.5 ml RNA Later (Applied Biosystems, Foster City, CA) prior to extraction. RNA was extracted using the SV Wizard Total RNA Isolation kit (Promega, Madison, WI). RNA was routinely checked for DNA contamination via 16S PCR as described above. DNase I (Fermentas, Glen Burnie, MD) treatment of RNA was performed as required. Briefly, 1 μg of RNA was digested with 1U DNase I (Fermentas, Glen Burnie, MD) in a reaction containing 1x reaction buffer (Fermentas) and Nuclease-free H_2O (Ambion, Applied Biosystems, Foster City, CA). The reaction mixture was incubated at 37^{o}C for 55 minutes and subsequently incubated with 1U DNase Stop Solution (Promega, Madison, WI) at 65^{o}C for 10 minutes. Reverse transcription of purified RNA was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

2.3.8 Quantitative reverse transcription PCR (qRT-PCR). qRT-PCR was performed in 30 μl reaction mixtures on a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA). Gene copy calculations were done as previously described (33). Standard curves were constructed using ten-fold serial dilutions of the target fragment. Triplicate cDNA samples were assayed for transcription of each target gene. qRT-PCR reaction mixtures (30 μl) contained 0.2 μM each forward and reverse primers (KOdenAF/R or rpoB1698F/2041R ((9), Table 2.2) and 1X SYBR Green PCR Master

Mix (Applied Biosystems, Foster City, CA) with a balance of Nuclease-free H_2O (Ambion, Applied Biosystems, Foster City, CA). qRT-PCR thermocycling monitoring *denA* began with Stage 1(50^{o}C, 2 min), followed by Stage 2 (95^{o}C, 10 min). Stage 3 included forty repetitions of Step 1 (95^{o}C, 15 sec), Step 2 (57^{o}C, 60 sec), and Step 3 (72^{o}C, 60 sec). Data collection occurred during Stage 3, Step 1. A final dissociation stage included 95^{o}C (15 sec), 60^{o}C (60 sec) and finally 95^{o}C (15 sec). qRT-PCR conditions for *rpoB* were identical to those for *denA*, except there were 35 repetitions of Stage 3 of Step 1 (94^{o}C, 30 sec), Step 2 (50^{o}C, 90 sec), and Step 3 (72^{o}C, 90 sec). Efficiency for all qRT-PCR reactions were greater than 70% (slope between -3.61 and -4.27) and R^2 values for all qRT-PCR reactions were greater than 0.99.

2.3.9 Identification of *denA*. Initial identification of *denA* was accomplished using degenerate primers (DengenF and DengenR4, Table 2.2) and PCR as described above, except for the addition of 1 μl of dimethyl sulfoxide per 20 μl reaction (Table 2.2). The purified amplicon was sequenced by Nevada Genomics Center (Reno, NV) and primers were designed for primer walking (20). Primer walking using CEKG2A, B and C along with CEKG4 (Table 2.2) was performed as previously described (18) using 5X Phusion High Fidelity PCR Master Mix with GC Buffer (New England Biolabs, Ipswich, MA). The open reading frame (ORF) of *denA* was identified by an online ORF finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

2.3.10 Alignments and phylogenetic trees. Highly related sequences of DenA were identified by BLASTP analysis (http://www.ncbi.nlm.nih.gov) (2). Nucleotide and

amino acid sequences were obtained from NCBI. Alignments were conducted in Bioedit using Clustal W (EBI, (45)). Phylogenetic trees were constructed with MEGA4 using the neighbor joining method and bootstrap analysis (500 replicates) (14, 34, 41). Protein families and conserved regions were identified using the Pfam (http://pfam.sanger.ac.uk/) and CDD (24) databases (5, 43, 44). Evolutionary distances for proteins were computed using the Poisson correction method (50). Evolutionary distance for nucleic acids were computed using the Maximum Composite Likelihood Method (42). Redundant and hypothetical results were omitted prior to final tree construction.

2.3.11 Insertional mutagenesis of *denA* from *Variovorax* sp. JS669. A partial fragment of *denA* from *Variovorax* sp. JS669 was amplified using primers KOdenAF and KOdenAR (Table 2.2), purified (Qiaquick PCR purification kit, Qiagen) and cloned into pVIK110 (21) using EcoRI and SalI restriction sites (New England Biolabs, Ipswich, MA) and T4 DNA ligase (New England Biolabs, Ipswich, MA). The *denA*:pVIK110 construct was electroporated into electrocompetent *E. coli* S17-1 λ-pir cells (21) (2.5 kV, 25 μF, 200 Ω). Electroporated cells were flushed with 900 μl of Super Optimal Broth with catabolite repression (SOC) media and incubated at 37^{o}C for 2 hours. Cells were recovered on LB with 1.5% agar supplemented with kanamycin (30 μg/ml) (21, 36). The resulting *E.coli* clone (*denA*:pVIK110: *E.coli* S17-1) was conjugated with *Variovorax* sp. JS669 as previously described (19, 21). Conjugants were recovered in BLK, glucose (5 mM), NH_4Cl (1 mM) and carbenicillin (100 μg/ml) for six hours at 30^{o}C and selected on 1.5% agar plates containing BLK, glucose (5mM), NH_4Cl (1 mM), carbenicillin (100 μg/ml) and kanamycin (30 μg/ml). Correct insertion of the suicide

vector was confirmed via PCR using primers KOdenAF and LacZR (19) for amplification and 502F (Table 2.2) for sequencing through Genewiz (New Jersey).

2.3.12 Test of HNPA denitrase clone constructs. pBAD plasmid constructs including the putative *denA* of *Variovorax paradoxus* JS171 (designated pJS337) and an unknown, but phylogenetically similar FAD monooxygenase of *Bordetella bronchiseptica* RB50 (designated pJS338) were assayed for their ability to release nitrite from HNPA. Single colonies from the clones pJS337 or pJS338 were inoculated into LB medium supplemented with ampicillin (100 μg/ml) and IPTG (200 μg/ml) and then grown for 17 hours at 30°C (36). The culture was harvested by centrifugation to remove the supernatant, washed once in phosphate buffer (20 mM; pH 7.0), then suspended in phosphate buffer containing HNPA (100 μM). The reaction mixture was shaken at 30°C and samples were taken at intervals to measure the nitrite concentration as described above.

2.3.13 Chemicals. 3NTyr was obtained from Cayman Chemical (Ann Arbor, MI). HNPA was from Sigma-Aldrich (Milwaukee, WI).

2.4 RESULTS

2.4.1 Isolation and Identification of *Variovorax* sp. JS669. Ten isolates were obtained via selective enrichment with 3NTyr. All of the 3NTyr-degrading isolates demonstrated

nitrite release when grown on either 3NTyr (500 μM) or HNPA (500 μM), confirming that HNPA denitration occurred similarly to the published 3NTyr biodegradation pathway. Based on ease of growth with 3NTyr (500 μM) as compared to the other isolates, one isolate was chosen for further study. BLAST analysis of the sequenced 16S sequence amplicon from the isolates revealed 97 % identity with *Variovorax paradoxus* S110, so we designated the isolate *Variovorax* sp. JS669.

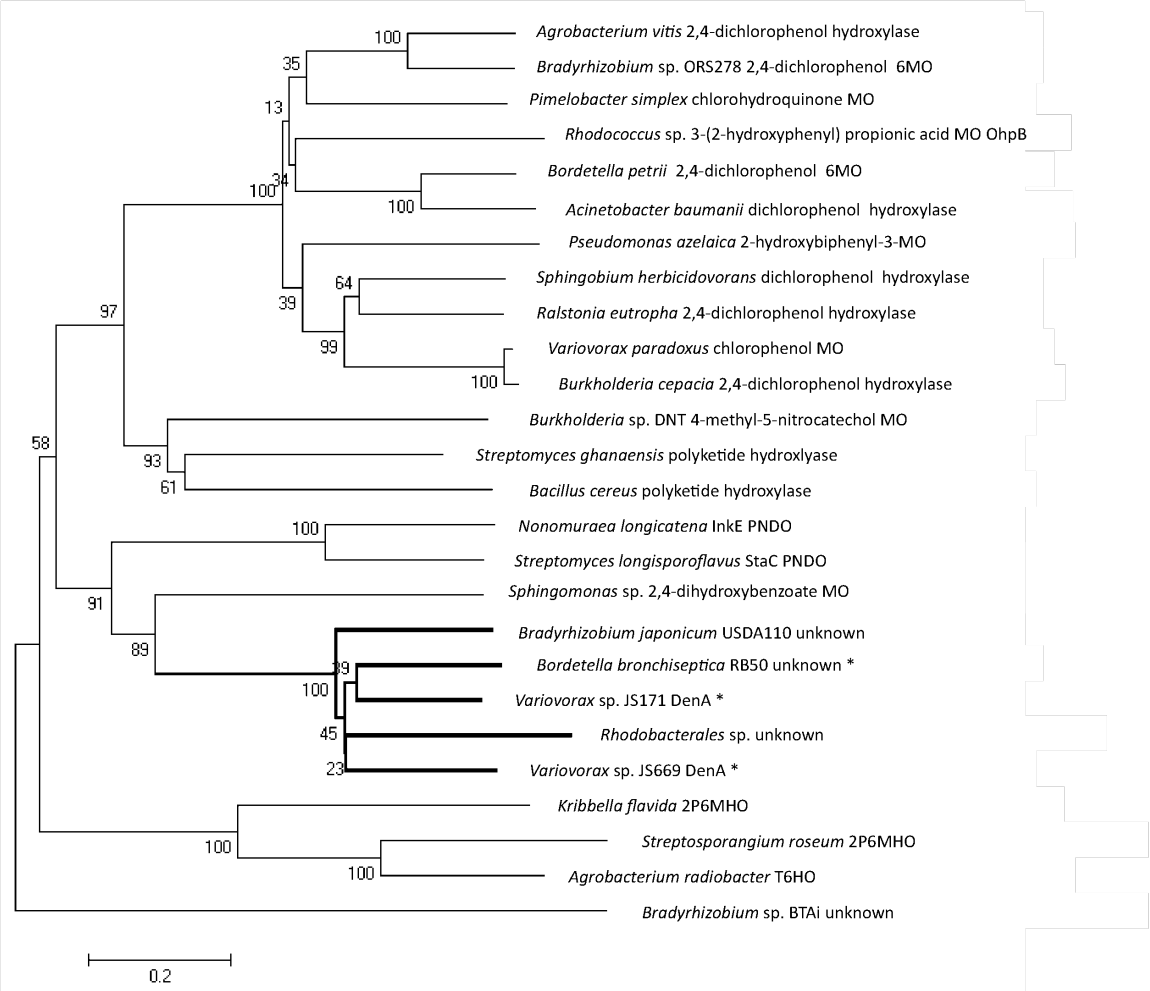
2.4.2 Respirometry. Stimulation of oxygen uptake by 3NTyr, HNPA, and homoprotocatechaute (HPC) in 3NTyr-grown cells, but not in glucose-grown control cells, indicated that the enzymes catalyzing transformation of all three compounds were inducible. The stoichiometry of HNPA-dependent oxygen uptake was 1 mol greater than that of HPC, which indicated that a monooxygenase is involved in the denitration of HNPA to HPC (27). HPC oxidation catalyzed by cell extracts required 0.95 mol O_2/ mol HPC as previously reported (22), suggesting that the homoprotocatechuate ring-cleavage dioxygenase was responsible for the reaction (Table 2.3) (27). Therefore, the degradation pathway of 3NTyr in *Variovorax* sp. JS669 was consistent with what we reported previously for *Burkholderia* sp. JS165 and *Variovorax paradoxus* JS171 (27).

Testing substrates	Oxygen uptake (nmol O_2/min/mg of protein) by cells grown in	
	3-Nitrotyrosine	Glucose
3-Nitrotyrosine	30.3 ± 1.9 (2.78)[a]	ND
4-Hydroxy-3-nitro-phenylacetate	98.8 ± 5.1 (2.9)	ND
Homoprotocatechuate[a]	40.8 ± 5.1 (2.08)	ND
Glucose	NT	5.2±0.42

Table 2.3. O_2 uptake by 3NTyr or glucose grown cells. Reaction mixtures contained 1.8 ml of oxygen-saturated phosphate buffer (0.02 M; pH 7.0; 25°C), cells (0.46 mg/ml protein for induced cells, 0.6 mg/ml protein for uninduced cells) and test substrates (20 μM). ND: Not detected; NT: Not tested.

[a]Stoichiometries in parentheses (mol O_2/mol substrate).

2.4.3 Identification of DenA. The amplicon obtained using DengenF/R4 was sequenced and the translated amino acid sequence exhibited high similarity to the previously identified DenA from *Variovorax* paradoxus JS171 (30). Primer walking revealed the entire JS669 *denA* sequence. A group of FAD monooxygenases of unknown function exhibited high identity with JS669 DenA by BLASTP analysis (2). *Variovorax* sp. JS669 DenA and *Variovorax paradoxus* JS171 putative DenA along with the *Bordetella bronchiseptica* RB50 and other uncharacterized FAD monooxygenases, form a distinct clade separate from other FAD monooxygenases (Figure 2.2). Cells of *Variovorax paradoxus* JS171, *Variovorax* sp. JS669 and an *E.coli* clone expressing the *B. bronchiseptica* RB50 FAD monooxygenase catalyze the release of nitrite from HNPA. It is thus possible that the clade of FAD monooxygenases including DenA includes enzymes specific for denitration of nitroarenes.



2P6MHO = 2-polyprenyl-6-methoxyphenol hydroxylase-like-oxidoreductase
FAD DO = FAD-dependent oxidoreductase
T6HO = oxygenase(tetracycline-6-hydroxylase)
MO = Monooxygenase

Figure 2.2. FAD monooxygenase phylogenetic tree displaying the DenA clade. An amino acid multiple sequence alignment was constructed of the top 250 BLAST matches to DenA using Bioedit. The results were used to construct a Neighbor-joining phylogenetic tree using MEGA4 (41). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (14). The tree is drawn to scale, with branch lengths in the same units as

those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (50) and are in the units of the number of amino acid substitutions per site. The DenA clade is demarcated in bold. Biochemically characterized DenA are shown (*). Triangles represent novel isolates described in this study. Accession numbers are as follows: *Bordetella bronchiseptica* RB50 (NP 887282.1), *Bradyrhizobium japonicum* USDA 110 (NP 767049.1), *Rhodobacterales* bacterium HTCC2654 (ZP 01015364.1), *Kribbella flavida* (ZP 03861503.1), *Streptosporangium roseum* (ZP 04477043.1), *Bradyrhizobium* sp. BTAi (YP 001237466.1), *Agrobacterium radiobacter* (YP 002545402.1), *Agrobacterium vitis* (YP 002539792.1), *Bordetella petrii* (YP 001629936.1), *Pimelobacter simplex* (ABA61847.1), *Acinetobacter baumannii* (ABO11509.2), *Bradyrhizobium* sp. ORS278 (YP 001204343.1), *Sphingobium herbicidovorans* (CAF32816.1), *Rhodococcus* sp. OhpB (AAF81824.1), *Variovorax_paradoxus* (BAA88071.1), *Pseudomonas azelaica* (AAB57640.1), *Ralstonia eutropha* (YP_025383.1), *Burkholderia cepacia* (AAB86805.1), *Streptomyces ghanaensis* (ZP 04689828.1), Nonomuraea longicatena (ABD59214.1), *Streptomyces longisporoflavus* (ABI94390.1), *Bacillus cereus* (ZP 00235674.1), *Sphingomonas* sp. (CAA51370.1), *Burkholderia* sp. DNT (ABC00744.1).

DenA of JS669 was aligned with other FAD monooxygenase sequences from the HNPA denitrase clade to identify conserved motifs (Figure 2.3). The FAD binding fingerprints 1 and 2, along with a dual FAD/NAD(P)H binding site were identified in *Variovorax* sp. JS669 and *Variovorax paradoxus* JS171, along with the eight other FAD monooxygenases (13). In addition, DenA from *Variovorax* sp. JS669 was separately

compared with enzymes that catalyze the elimination of nitro-, chloro- and fluoro- groups from phenolic compounds (Figure 2.4). DenA enzymes clustered and there were several conserved residues among the denitration, dechlorination and defluorination enzymes that were aligned (Figure 2.5).

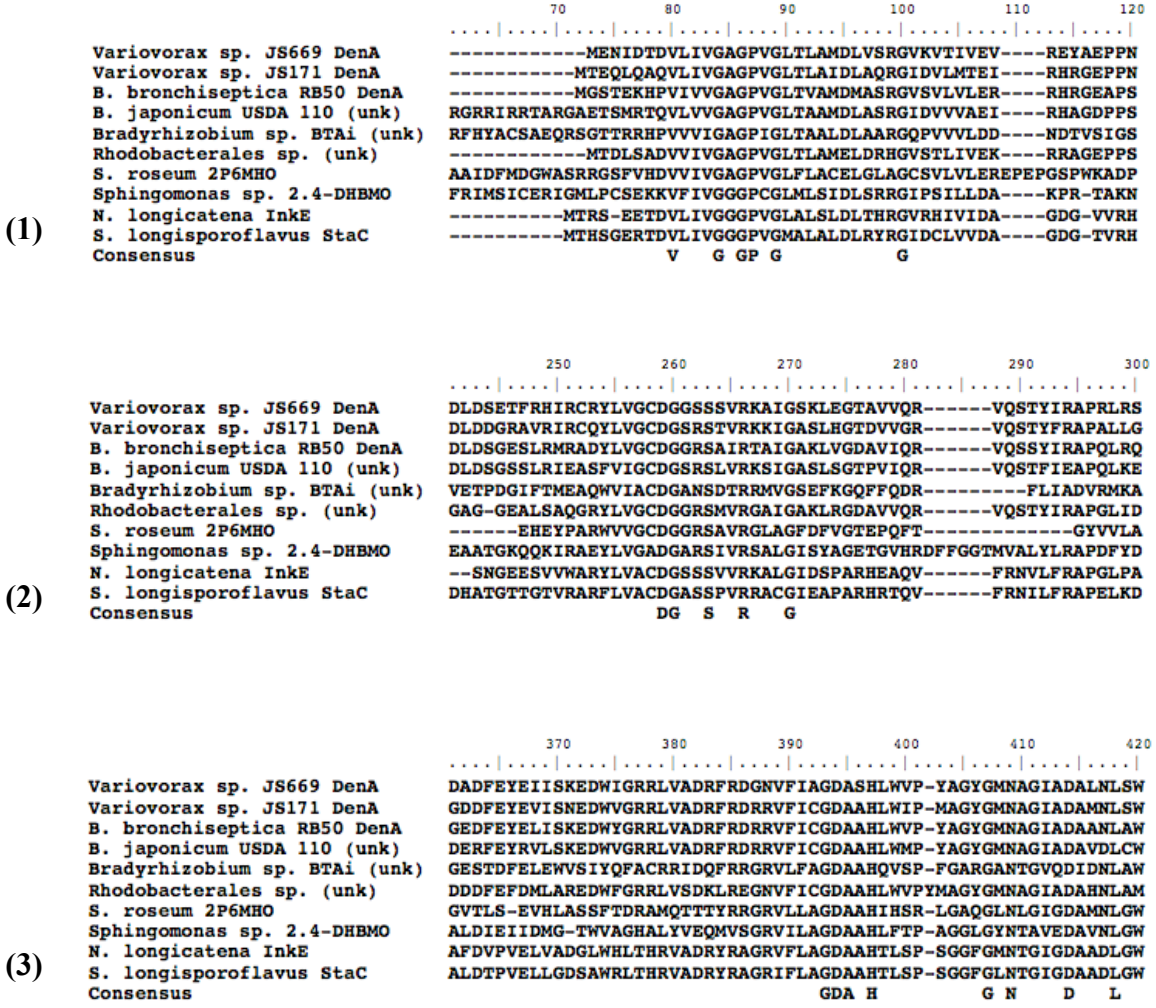


Figure 2.3. Conserved FAD and NAD(P)H binding domains among DenA and closely related FAD monooxygenases. All of the functional and putative DenA sequences from the HNPA denitrase clade were used to make a second MSA, along with several representative FAD monooxygenases. Conserved residues within consensus

sequences are shown below. Conserved residues in region (1) are part of the βαβ FAD binding domain, fingerprint 1, conserved residues in region (2) are part of the FAD/NAD(P)H dual binding site and conserved residues in region (3) are part of the FAD binding domain, fingerprint 2, which includes the NAD(P)H binding site GxGxxA (15).

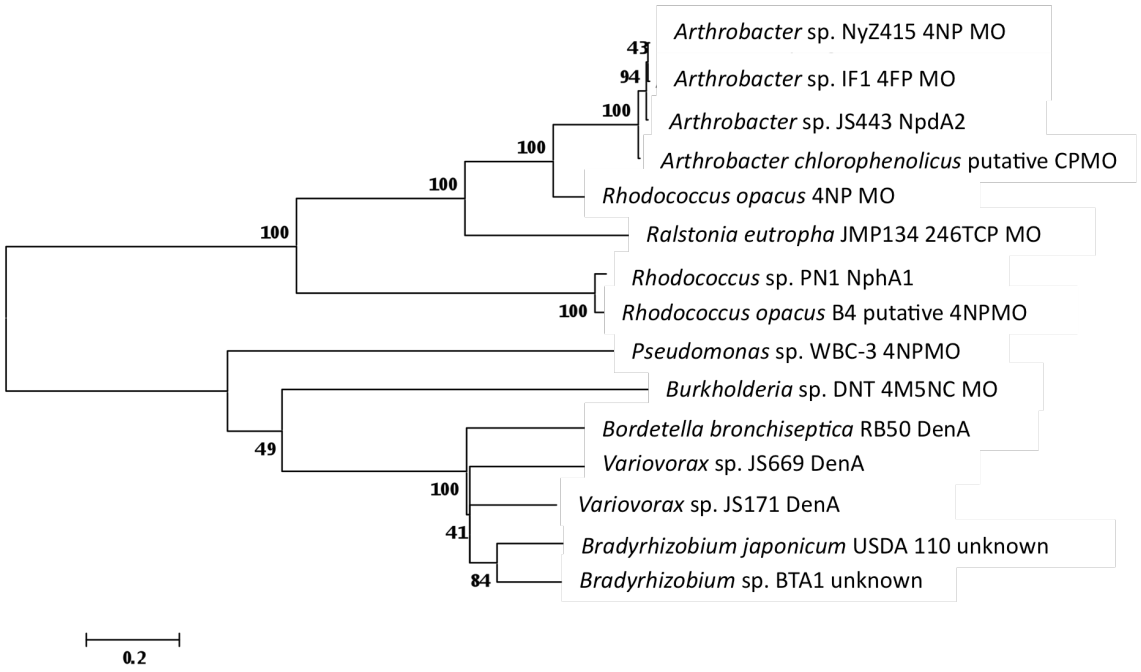
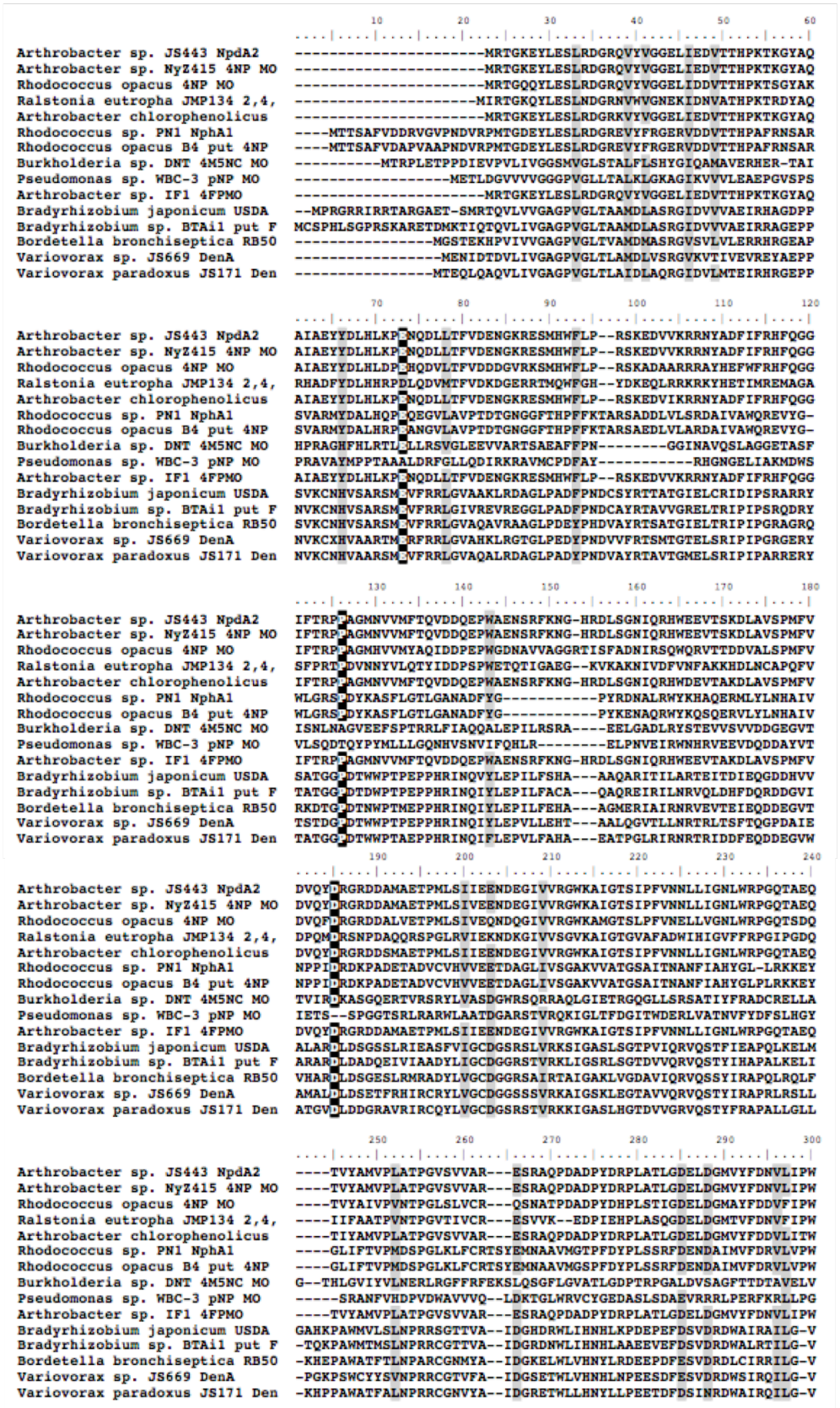
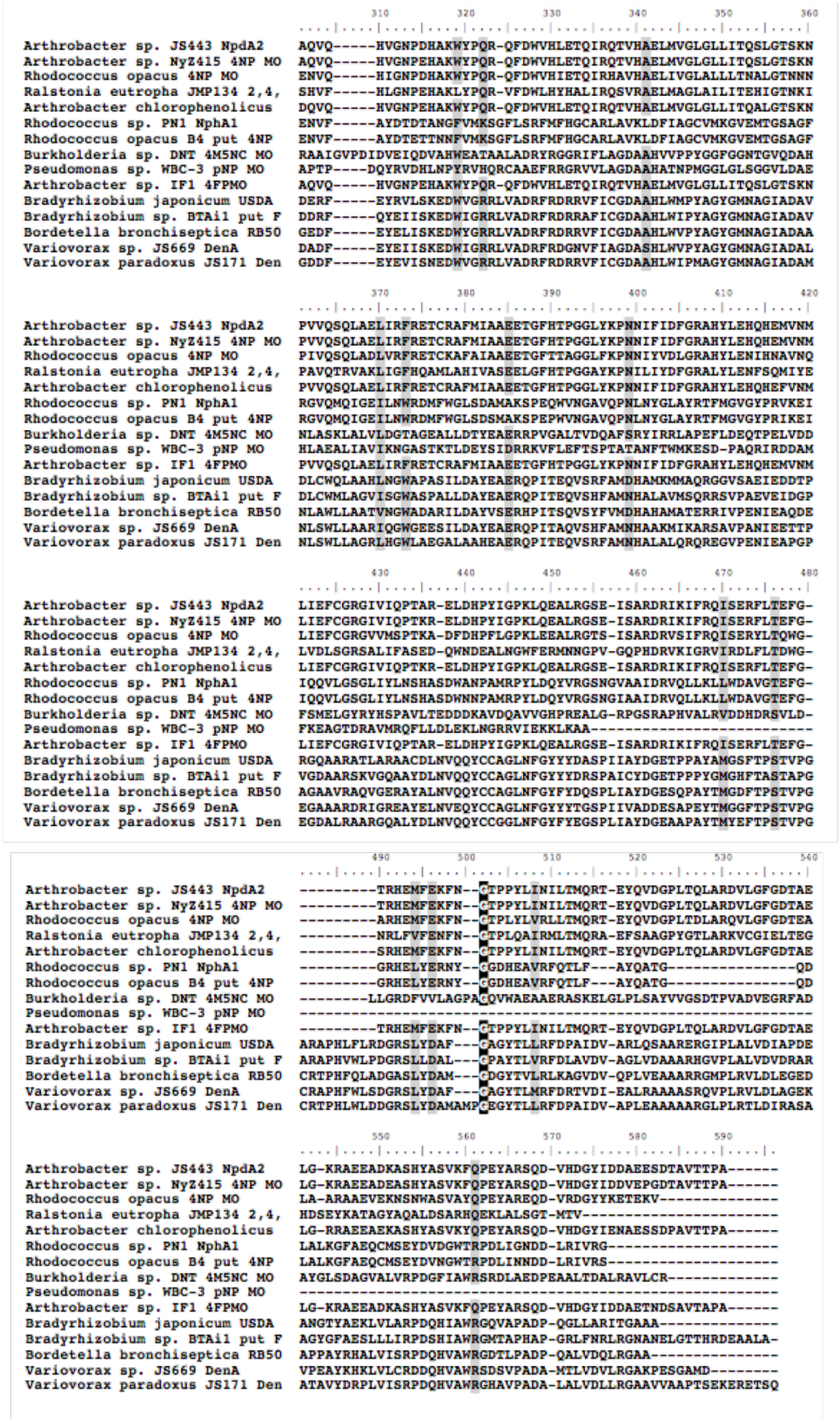


Figure 2.4. Phylogenetic tree of *Variovorax* sp. JS669 DenA with enzymes that catalyze denitration, dechlorination and defluorination. Accession numbers are *Arthrobacter* sp. JS443 NpdA2 (ABL75143.1), *Arthrobacter* sp. NyZ415 (ACX85440.1), *Rhodococcus opacus* 4-nitrophenol monooxygenase oxygenase component (BAD30042.1), *Ralstonia eutropha* JMP134 2,4,6-trichlorophenol monooxygenase (AAM55214.1), *Arthrobacter chlorophenolicus* putative chlorophenol monooxygenase large subunit (AAN08754.1), *Rhodococcus* sp. PN1 NphA1

(BAB86378.2), *Rhodococcus opacus* B4 putative 4-nitrophenol hydroxylase component A (YP 002780933.1), *Burkholderia* sp. DNT 4-methyl-5-nitrocatechol monooxygenase (ABC00744.1), *Pseudomonas* sp. WBC-3 *para*-nitrophenol 4- monooxygenase (ABU50908.1), *Arthrobacter* sp. IF1 oxygenase component of 4-fluorophenol monooxygenase (BAI53131.1), *Bradyrhizobium japonicum* USDA 110 putative FAD monooxygenase (BAC45674.1), *Bradyrhizobium* sp. BTAi FAD-binding monooxygenase (ABQ36142.1), *Bordetella bronchiseptica* RB50 FAD monooxygenase (CAE31232.1).


Arthrobacter sp. JS443 NpdA2
Arthrobacter sp. NyZ415 4NP MO
Rhodococcus opacus 4NP MO
Ralstonia eutropha JMP134 2,4,
Arthrobacter chlorophenolicus
Rhodococcus sp. PN1 NphA1
Rhodococcus opacus B4 put 4NP
Burkholderia sp. DNT 4M5NC MO
Pseudomonas sp. WBC-3 pNP MO
Arthrobacter sp. IF1 4FPMO
Bradyrhizobium japonicum USDA
Bradyrhizobium sp. BTAi1 put F
Bordetella bronchiseptica RB50
Variovorax sp. JS669 DenA
Variovorax paradoxus JS171 Den

(A)


Arthrobacter sp. JS443 NpdA2
Arthrobacter sp. NyZ415 4NP MO
Rhodococcus opacus 4NP MO
Ralstonia eutropha JMP134 2,4,
Arthrobacter chlorophenolicus
Rhodococcus sp. PN1 NphA1
Rhodococcus opacus B4 put 4NP
Burkholderia sp. DNT 4M5NC MO
Pseudomonas sp. WBC-3 pNP MO
Arthrobacter sp. IF1 4FPMO
Bradyrhizobium japonicum USDA
Bradyrhizobium sp. BTAi1 put F
Bordetella bronchiseptica RB50
Variovorax sp. JS669 DenA
Variovorax paradoxus JS171 Den

(B)

Figure 2.5. Alignment of denitrases with enzymes catalyzing nitro-, chloro- and fluoro-group removal from aromatic compounds. (A) Residues 1-300, (B) Residues 301-596. Residues conserved at 85% are highlighted in grey, whereas more highly conserved residues are marked in black. Conserved residues 33, 39, 41, 46 and 49 are part of the FAD binding domain fingerprint 1, conserved residues 203 and 209 are part of the FAD/NAD(P)H dual binding site and conserved residue 341 is part of the FAD binding domain fingerprint 2.

2.4.4 Transcription of *denA*. Quantitative reverse transcription PCR (qRT-PCR) was conducted to determine whether transcription of *denA* was inducible. RNA extracted from log phase cultures grown with either 3NTyr or glucose as the sole carbon source was reverse transcribed into cDNA for use in qRT-PCR. Transcription of *denA* and *rpoB* under induced (3NTyr-grown) and uninduced (glucose-grown) conditions and *rpoB* revealed differential expression between the two genes. There was a 19.5-fold (standard deviation of 3.11) upregulation of *denA* (Slope = -3.61, $R^2 = 0.999$) compared to the endogenous control (*rpoB*; Slope = -4.27, $R^2 = 0.996$) in *Variovorax* sp. JS669 when cells were grown on 3NTyr but not on glucose. Inducible transcription of *denA* correlates well with oxygen uptake results (Table 2.3) and with previously reported observations that the 3NTyr biodegradation pathway is inducible (27).

2.4.5 Loss of function mutant analysis. To rigorously establish the role of DenA in 3NTyr degradation, an insertional mutant in *Variovorax* sp. JS669 *denA* was constructed and confirmed via sequencing. JS669*denA*::pVIK110 grows at a greatly reduced

efficiency on 3NTyr as the sole carbon source, taking triple the amount of time as the wildtype organism to degrade 3NTyr, and completely lost the ability to grow on HNPA (Figure 2.6). HPLC analysis of culture fluid during growth of JS669*denA*::pVIK110 on 3NTyr showed scant accumulation of HNPA, but substantial accumulation of two other unknown peaks at retention times of 5.2 and 5.5. Given the 3NTyr degradation pathway (27), the mutant probably gains nitrogen from 3NTyr metabolism, but would be unable to obtain carbon through the same metabolic pathway. It is thus possible that JS669*denA*::pVIK110 uses an alternate pathway, such as one not outlined in Figure 2.1, to obtain carbon from 3NTyr. The results of experiments with the mutant indicate that *denA* is responsible for metabolism of HNPA and essential for the complete degradation of 3NTyr in the wildtype JS669.

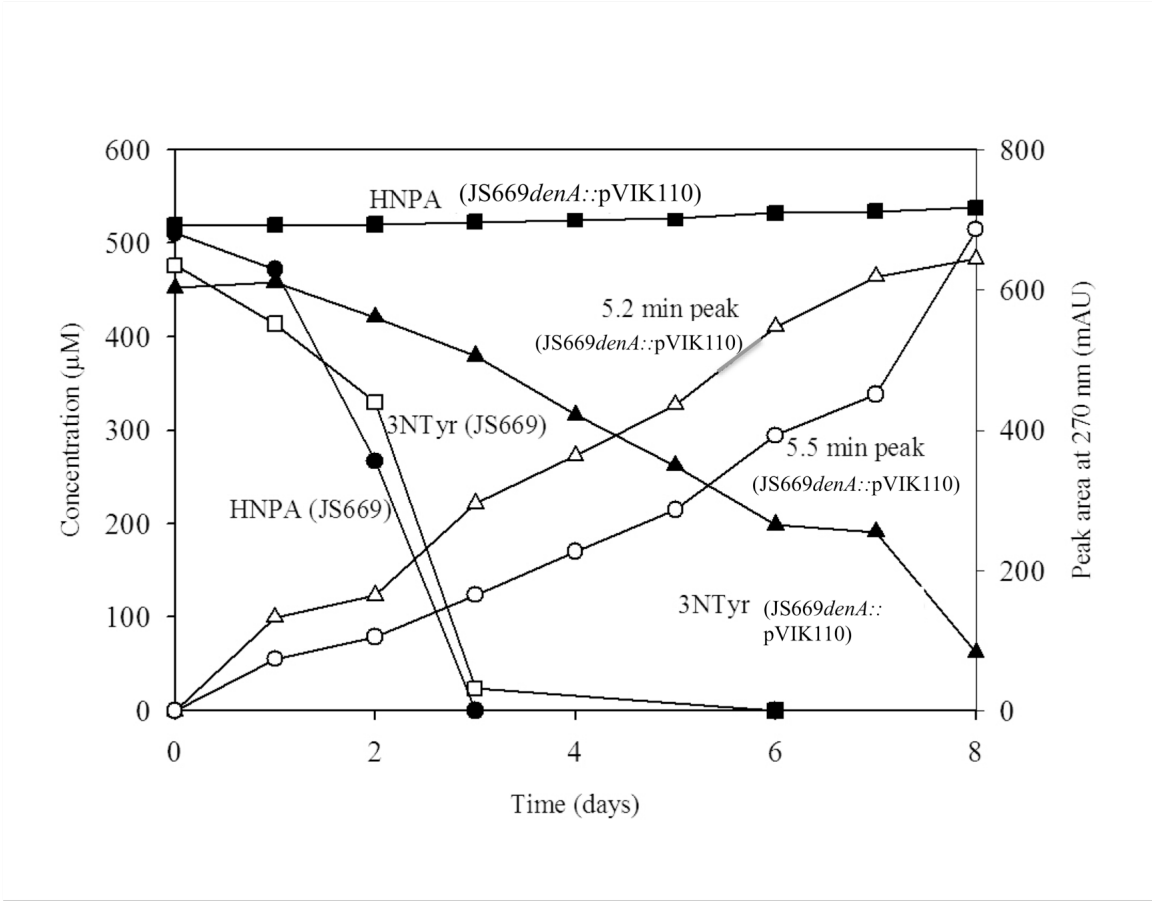


Figure 2.6. Transformation of 3NTyr and/or HNPA by *Variovorax* sp. JS669 and JS669*denA*::pVIK110. ●, Degradation of HNPA by wild-type JS669; □, degradation of 3-NTyr by wild-type JS669; ▲, degradation of 3-NTyr by the JS669*denA*::pVIK110 mutant; ■, degradation of HNPA by the JS669*denA*::pVIK110 mutant; Δ, accumulation of 5.5 min retention time peak by the JS669*denA*::pVIK110 mutant; ❍, accumulation of 5.5 min retention time peak by the JS669*denA*::pVIK110 mutant. (Typical results presented).

2.4.6 *In vivo* analysis of DenA. In order to determine the function of putative FAD monooxygenases that were highly similar to JS669 DenA, the genes from two other microbes were heterologously expressed in *E. coli* (obtained from Ray Payne) (30). Such clones were tested for the ability to transform HNPA and release nitrite. Nitrite was detected from clones pJS337 and pJS338, concomitant with HNPA disappearance. Thus, both the putative DenA from *Variovorax paradoxus* JS171 and the uncharacterized FAD monooxygenase from *B. bronchispetica* RB50 catalyze HNPA denitration.

2.4.7 Auxanography with *Bradyrhizobium* strains. Auxanography experiments were conducted to determine whether strains that contained putative FAD-monooxygenases similar to JS669 DenA could grow on 3NTyr or HNPA. Neither *Bradyrhizobium japonicum* USDA110, nor *Bradyrhizobium* sp. BTAi grew on auxanography plates with either 3NTyr or HNPA.

2.5 DISCUSSION

Three lines of evidence rigorously identify *denA* of *Variovorax* sp. JS669 as the gene encoding the key enzyme for 3NTyr degradation. First, the 3NTyr degradation pathway in JS669 is inducible, as is the previously described pathway (27). Next, *denA* is upregulated when grown on 3NTyr. Finally, JS669*denA*::pVIK110 could not catalyze HNPA denitration. Together the mutant and qRT-PCR experiments establish that DenA is the functional HNPA denitrase in *Variovorax* sp. JS669. Many attempts were

conducted to identify DenA activity in crude cell extracts, but no denitrase activity was detected (Parks *et al*, unpublished). Additionally, several clones to heterologously express the JS669 DenA in *E. coli* and *Pseudomonas aeruginosa* PAO1C were constructed, but all attempts to express DenA failed. It is possible that cracking the cells to prepare for experiments with crude cell extracts in some way damages the protein or another component required for the expression of DenA. Also, *denA* in JS669 uses many rare codons for expression, which may contribute to the failure to express the protein. Finally, expression of *denA* could have requirements that a heterologous host might be unable to provide.

Variovorax sp. JS669 DenA is composed of several conserved motifs that are important for both FAD and NAD(P)H binding (Table 2.4). The GD motif, part of the FAD binding domain fingerprint 2, was contained in all of the aligned sequences and is hypothesized to be involved in the hydrogen bonding to the 3'-hydroxyl of the FAD ribose structure in several enzymes, including the rubredoxin reductase in *Pseudomonas oleovorans* (11, 13, 16). The DG motif, found within a dual FAD/NAD(P)H binding site was also present in all aligned sequences and has two functions in FAD monooxygenases. It is involved in recognition of NAD(P)H and with indirect binding of the FAD pyrophosphate (13). The FAD binding fingerprint 1 in all of the aligned sequences included the characteristics of the Rossman βαβ fold (13). The second NAD(P)H binding site in fingerprint 2, GxGxxA was identified in seven of the aligned sequences and was 100% conserved among all DenA and FAD monooxygenase sequences in the HNPA denitrase clade (32). Identification of the conserved motifs in the DenA of JS669 reveal that the enzyme is an NAD(P)H-dependent FAD monooxygenase.

When *Variovorax* sp. JS669 DenA was aligned with other enzymes that denitrate, dechlorinate or defluorinate such as *p*-nitrophenol monooxygenase from *Pseudomonas* sp. WBC-3 and NpdA2 from *Arthrobacter* sp. JS443, there were thirty-five amino acid residues that were 85% conserved among all of the enzymes (Table 2.5). Conserved residues spanned the length of the peptides. Eight of the conserved residues were within the FAD and NAD(P)H conserved domains described above; however the remaining twenty-seven residues are seemingly unrelated to conserved domains for FAD and NAD(P)H binding. It is possible that such residues confer the ability to preferentially attack a nitro-, or other chloro- or fluoro- moiety, on an aromatic compound. Hence, at least some of such residues may be involved with substrate binding. The *Pseudomonas putida* DLL-E4 PnpA crystal structure was recently solved (23), however the active site remains unknown. Further studies into denitrase structures will reveal the role of the nineteen conserved residues and elucidate the role of such residues in denitration, dechlorination and defluorination.

There are several distinct classification systems of FAD monooxygenases, typically based on cofactor and coenzyme usage, conserved sequences and gene organization (28, 32, 38). Based on the single Rossmann fold binding domain, the GxGxxG, DG and GD conserved binding motifs and the sequence similarity to previously characterized FAD monooxygenases, we conclude that DenA is a single component Class A flavoprotein monooxygenase (13, 28, 32, 38, 48). Class A monooxygenases typically catalyze *ortho*- or *para*- hydroxylation with aromatic substrates that contain an activating hydroxyl or amino group (38, 48).

Catalytic capabilities of FAD monooxygenases have been well-studied, and the specific chemical reactions catalyzed by the enzymes are widespread and varied (38, 39). The *Variovorax* sp. JS669 DenA is the first identified HNPA denitrase, representative of a novel clade of similar enzymes. The fact that several other members of the clade can also remove the nitro group from HNPA indicates that the ability is widespread. There are many natural analogs of both 3NTyr and HNPA, including 4-hydroxy-3-nitro-phenylethylamine (26), 3'-nitrogenistein (1) and 4-hydroxy-3-nitrobenzeneacetic acid (40) produced by plants, bacteria and fungi respectively. We thus hypothesize that members of the DenA clade that do not catalyze denitration of HNPA might instead catalyze denitration of the natural analogs of 3NTyr or HNPA.

The experiments described in this report have revealed a new group of FAD monooxygenases. Analysis of the cloned gene for the *B. bronchiseptica* RB50 FAD monooxygenase permitted the functional annotation of a gene product of previously unknown function. Further characterization of the enzymes in the DenA clade will reveal their physiological roles and whether they denitrate natural analogs to 3NTyr. Structure determination will be required to reveal key conserved residues required for denitration of aromatic compounds by DenA.

ACKNOWLEDGEMENTS

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CHAPTER 3

PHYLOGENY AND DISTRIBUTION OF 3-NITROTYROSINE DEGRADING BACTERIA AND THE GENE (*denA*) ENCODING THE KEY DENITRASE FOR DEGRADATION

3.1 ABSTRACT

3-Nitrotyrosine (3NTyr) in protein is a biological marker for both infection and inflammation in a wide range of organisms. Bacteria able to biodegrade 3NTyr have been isolated by selective enrichment from a variety of habitats. Wide distribution of 3NTyr degraders implies that there is a substantial flux of 3NTyr in natural habitats. 3NTyr-degrading microbes are phylogenetically diverse and include representatives of, α–, β–, and γ– *Proteobacteria*. To determine whether all of the isolates used the same biodegradation pathway, primers were designed to amplify 4-hydroxy-3-nitro-phenylacetate (HNPA) denitrase genes (*denA*). 50% of our 3NTyr-degrading isolates produced a product that was similar to *denA*. The remaining isolates either have divergent *denA* or degrade 3NTyr through an alternate pathway. Identification and characterization of the diversity of 3NTyr-degrading bacteria and DenA will enable further understanding of the ecological role of 3NTyr-degrading isolates as well as the study of DenA and DenA-like FAD monooxygenases.

3.2 INTRODUCTION

Both 3NTyr and 4-hydroxy-3-nitrophenylacetic acid (HNPA) are naturally produced and their biosynthesis is associated with host immune defenses. 3-Nitrotyrosine (3NTyr) is a naturally occurring nitro compound formed as a result of nitric oxide release during stress and immune responses in mammals, mollusks and plants (5, 8, 10, 14, 16, 21, 29). Nitric oxide is produced during immune responses in plants and animals (5, 8, 10, 14, 15, 29). Subsequently, in the presence of the superoxide anion, nitric oxide yields peroxynitrite ($ONOO^-$), which nitrates tyrosine residues in proteins (7, 9, 16, 20). 3NTyr is an indicator of disease of the hosts including cystic fibrosis, asthma and diabetes in humans (9, 16, 20). Additionally, tyrosine nitration can damage and alter protein function (20, 30). Finally, HNPA produced by ascomycete fungi and mustard plants has antibacterial activity against bacteria (24, 27).

Biodegradation of 3NTyr and HNPA enables bacteria to gain carbon, nitrogen and energy and thus provides a significant advantage for soil microbes. The biochemical pathway for 3NTyr degradation was previously described for *Variovorax paradoxus* JS171 and *Burkholderia* sp. JS165 (18). HNPA denitrase is the key enzyme involved in the removal of the nitro group from 3NTyr intermediates and its activity can be monitored by the release of nitrite during 3NTyr degradation. Removal of the nitro group from HNPA leads to the formation of homoprotocatechuate, which can then enter central metabolism (18). The HNPA denitrase is encoded by *denA*, and represents a novel group of FAD monooxygenases (Chapter 2).

Natural nitro compounds, including 3NTyr, are increasingly being identified in various ecosystems (1, 2, 6, 13, 23), however the ecological impact of the compounds and the organisms responsible for their synthesis and degradation are relatively unknown. Identification of the phylogenetic and DenA diversity among 3NTyr-degrading isolates will identify responsible microbes and the genes encoding the key enzymes for 3NTyr biodegradation. Bacterial maintenance of the catabolic pathway for 3NTyr suggests repeated exposure to the compound. Thus, the presence of specific enzymes dedicated to 3NTyr metabolism implies the importance of 3NTyr degradation in natural habitats.

Facile isolation of 3NTyr-degrading bacteria during recent studies (18) (Chapter 2) led to two questions. (1) What is the phylogenetic distribution of 3NTyr-degrading bacteria? (2) How similar are *denA* sequences among 3NTyr-degrading bacteria? Based on widespread synthesis of 3NTyr among animals and plants, it is likely that the ability to degrade 3NTyr is widespread among bacteria.

Here we report the phylogenetic distribution of 3NTyr-degrading isolates along with DenA phylogeny of a subset of the isolates. Comparison of DenA with homologous enzymes revealed that DenA represents a novel group of FAD monooxygenases (Chapter 2). Development of probes for *denA* based on sequences from 3NTyr-degrading isolates enabled the identification of more *denA* sequences, which will permit further characterization of the enzyme and conserved motifs that enable the denitration of nitro compounds.

3.3 MATERIALS AND METHODS

3.3.1 Isolation and growth of 3NTyr degrading bacteria. Soil and water (fresh and marine) samples were obtained from Georgia (Atlanta, Dawsonville, Savannah and Tifton), Virginia, Puerto Rico, Nevada (Las Vegas Strip) , California (Manhattan and Hermosa Beaches) , and Panama City, Florida (Figure 3.1). Such samples were obtained from a wide variety of habitats including dinitrotoluene-contaminated soil, areas surrounding livestock housing facilities, agricultural fields, forested areas and oyster beds. Isolation of 3NTyr-degrading bacteria was conducted by selective enrichment with 3NTyr as the sole carbon, nitrogen and energy source as previously described (18). Cultures were maintained on agar plates containing BLK and 3NTyr (500 μM) as the sole carbon, nitrogen and energy source. Liquid cultures were grown in BLK containing 3NTyr (500 μM) both with and without glucose (5 mM) as an additional carbon source.

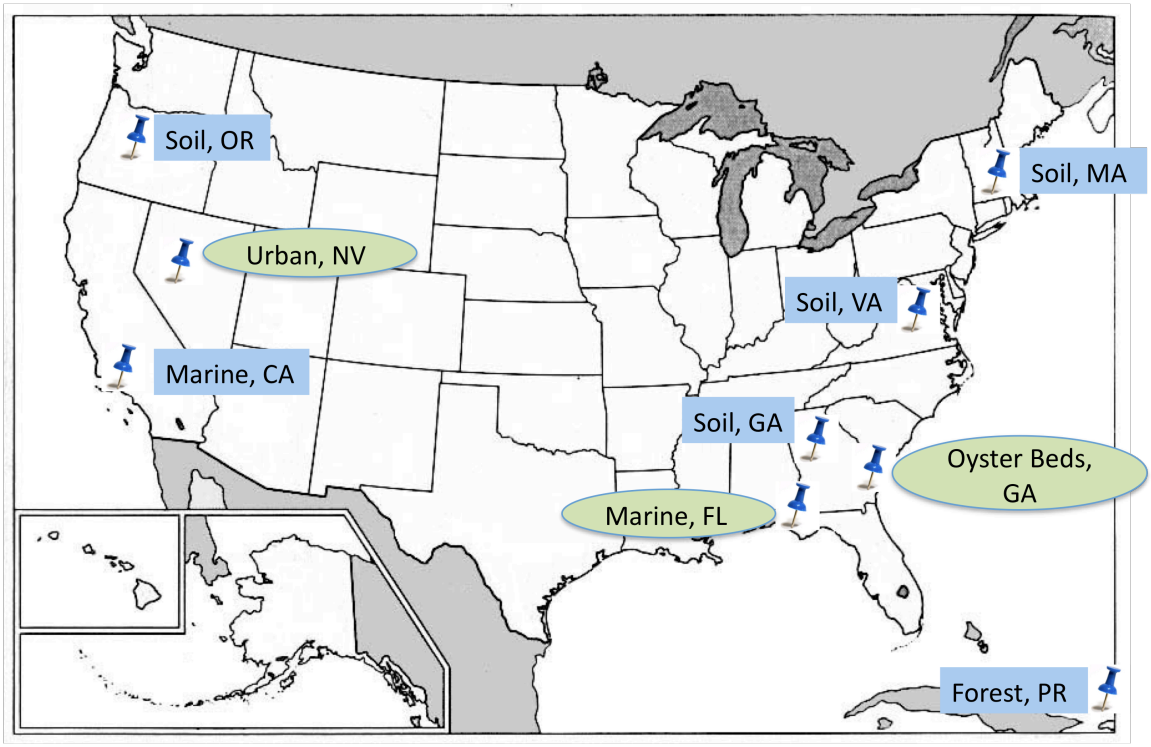


Figure 3.1. Map of sites sampled for 3NTyr degradation. Sites sampled for 3NTyr degradation. 3NTyr degradation was observed at all tested sites. 3NTyr-degrading isolates were obtained from the majority of sites (Rectangles). Some sites were not used to obtain 3NTyr-degrading isolates (Ovals).

3.3.2 Analytical Methods. The Griess reaction was employed to colorimetrically measure nitrite concentration as previously described (17). HPLC analyses to monitor 3NTyr and HNPA were conducted as previously described (18).

3.3.3 DNA extraction. Genomic DNA extractions were conducted using the SV Wizard Genomic DNA Purification kit according to printed instructions (Promega, Madison, WI).

3.3.4 Genome Sequencing of *Variovorax* sp. JS669. JS669 genomic DNA, JS165 genomic DNA (18) and JS171 fosmid 8F12 DNA containing DenA were sequenced by the Emory Georgia Research Alliance Genomic Center by 454 pyrosequencing. (http://www.corelabs.emory.edu/labs/gra_genome_center/index.html).

3.3.5 PCR amplification and DNA sequencing. Amplification of the partial 16S rRNA gene was conducted using primers 341F and 926R as previously described, with slight modification (Table 3.1) (4). Amplicons were purified using the Wizard SV Gel and PCR Clean up System (Promega, Madison, WI). Purified DNA was sequenced by Nevada Genomics Center (Reno, NV) or Genewiz (New Jersey). Sequences were analyzed by comparison with sequences in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/index.html) via BLASTN (16S rRNA gene) or BLASTP (DenA) analysis (3). All primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Primer	Sequence	Ta[1] (°C)	Time[a] (sec)	Time[e] (sec)	[$MgCl_2$] (mM)	Source
341F	5'- CCTACGGGIGGCIGCA	50	30	60	3.125	(4)
926R	5'- CCGICIATTIITTTIAGTTT	50	30	60	3.125	(4)
DenGenF	5'- AAYGTNAARTGYAAYCAYGT	43	30	60	7.5	(19)
DenGenR4	5'- GCRTTCATNCCRTANCCNGC	43	30	60	7.5	(19)
502F	5'- AGCGAGACCTTTCGGCATAT TCGT	56.8	60	60	2.5	This study
688R	5'- AGTAGTAGCACCATGAAGGC TTGC	56.8	60	60	2.5	This study
849R	5'- TTCGTACTCGAATCCGCATCG AC	56.8	60	60	2.5	This study

[1]Annealing Temperature, [a]Annealing Time and [e]Extension Time.

Table 3.1. Primers used in this study. PCR reactions contained template DNA along with total dNTPs (400 μM), combined forward and reverse primers (1 μM), $MgCl_2$, 1X GoTaq Flexi Buffer and 1U of Go Taq DNA Polymerase (Promega, Madison, WI). All PCR conditions began with an initial denaturation (95°C, 5 min) and continued with 35 cycles of denaturation (95°C, 2 min), primer annealing, and DNA extension. PCR cycles concluded with a final extension (72°C, 10 min). Degenerate nucleotide bases were used to increase the range of primer detection: Inosine (I) pairs with adenine (A), cytosine (C) or uracil (U); (Y) pairs with C or thymine (T); (N) pairs with A, C, guanine (G) or T; and (R) pairs with A or G.

3.3.6 Amplification of *denA* and development of *denA* specific primers. Amplification of the partial *denA* sequence was performed using primers DenGenF and DenGenR4 with PCR conditions similar to those for 16S rRNA PCR except for the addition of 1 μl of dimethyl sulfoxide per 20 μl reaction (Table 3.1) as previously described (Chapter 2).

Amplicons were purified and sent for sequencing as described above. Sequences that displayed high identity with FAD monooxygenase sequences were aligned for primer design using CLC Sequence viewer 6.1 (CLC Bio). Primers 502F, 688R and 849R were designed from highly conserved regions among the *denA* sequences from twelve isolates from Georgia, Virginia and Puerto Rico (Figure 3.2). Amplification of *denA* using a combination of 502F, 688R and 849R was performed using PCR conditions as described above for 341F/926R cycling (Table 3.1).

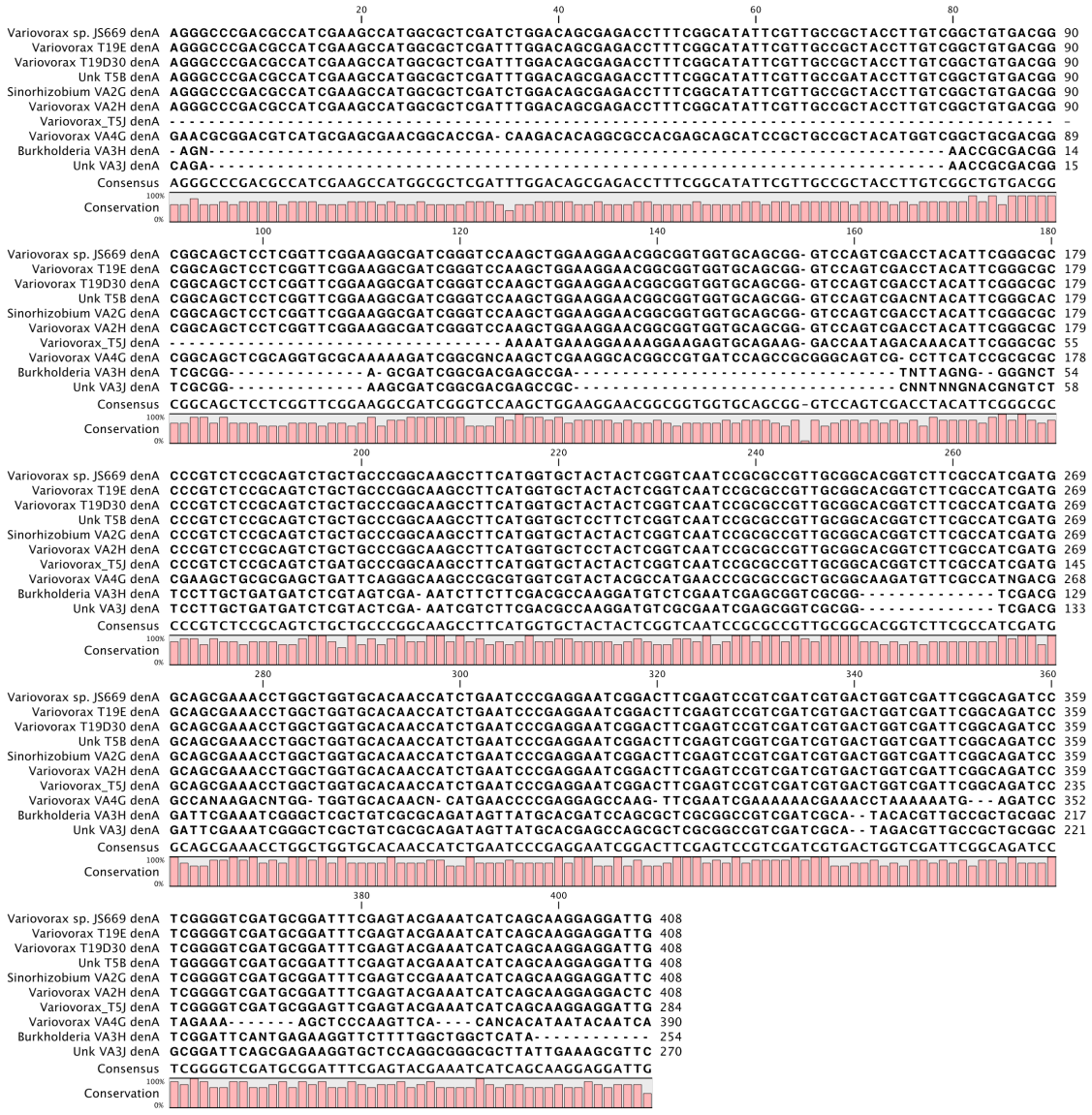


Figure 3.2. Multiple sequence alignment of DenA. Partial multiple sequence alignment (MSA) of *Variovorax* sp. JS669 with *denA* from nine other 3NTyr-degrading isolates from agricultural soil. Primers 502F (bases 41-64), 688R (bases 205-228) and 849R (bases 366-389) are displayed.

3.3.7 Alignments and phylogenetic trees. BLAST analysis was used to find related sequences (http://www.ncbi.nlm.nih.gov), and both nucleotide and amino acid sequences were obtained from the NCBI database (3). Gene context information of closely related

sequences was obtained from Integrated Microbial Genomics, DOE Joint Genome Institute (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi). Alignments were conducted in Bioedit using Clustal W (EBI) (28). Phylogenetic trees were constructed using MEGA4 using the neighbor joining method and bootstrap analysis (500 replicates) (12, 22, 25, 31). Evolutionary distances for nucleotide and protein sequences were computed using the Maximum Composite Likelihood method (26), and Poisson correction method (31) respectively.

3.4 RESULTS

3.4.1 3-Nitrotyrosine degradation in environmental samples. A wide variety of ecosystems were sampled to determine the diversity of 3NTyr degraders both by phylogeny and by type of habitat. In all environmental samples 3NTyr disappearance was detected by HPLC through several rounds of enrichment and transfer (Figure 3.1). Diverse microorganisms with the ability to degrade 3NTyr were isolated from soil obtained from agricultural facilities, forests, dinitrotoluene-contaiminated soil, and suburban and urban soil. 3NTyr-degrading isolates were most readily isolated from agricultural soil (Table 3.2).

Ecosystem Sampled	Number of sites tested within ecosystem	Number of isolates	Phylogeny of representative isolates	Number of DenA sequences identified
Suburban garden	3	10	*Microbacterium* sp.	2
Dinitrotoluene contaminated soil	1	4	*Microbacterium* sp., *Pseudomonas* sp.	0
Agricultural areas*	17	91	*Burkholderia* sp., *Delftia* sp., *Variovorax* sp., *Alcaligenes* sp., *Xanthomonas* sp., *Pseudomonas* sp., *Rhizobium* sp., *Xanthobacter* sp., *Afipia* sp.	12
Forested area	3	18	*Burkholderia* sp., *Variovorax* sp., *Alcaligenes* sp.	1

Table 3.2. Various ecosystems sampled and used for obtaining 3NTyr-degrading isolates.

3.4.2 16S rRNA characterization of 3NTyr-degrading isolates. To characterize 3NTyr-degrading isolates, representative 3NTyr-degrading bacteria isolated from enrichment cultures were identified via partial 16S rRNA amplification and sequencing. Based on BLAST analysis, the vast majority of isolates were gram negative and matched most closely with α–, β–, and γ– *Proteobacteria*. Two-gram positive isolates were most closely related to *Actinobacteria* (Figure 3.3). Such results reveal that 3NTyr-degrading bacteria are phylogenetically diverse as well as being found in diverse habitats (Table 3.2). It is thus apparent that 3NTyr is a common growth substrate used by soil microbes.

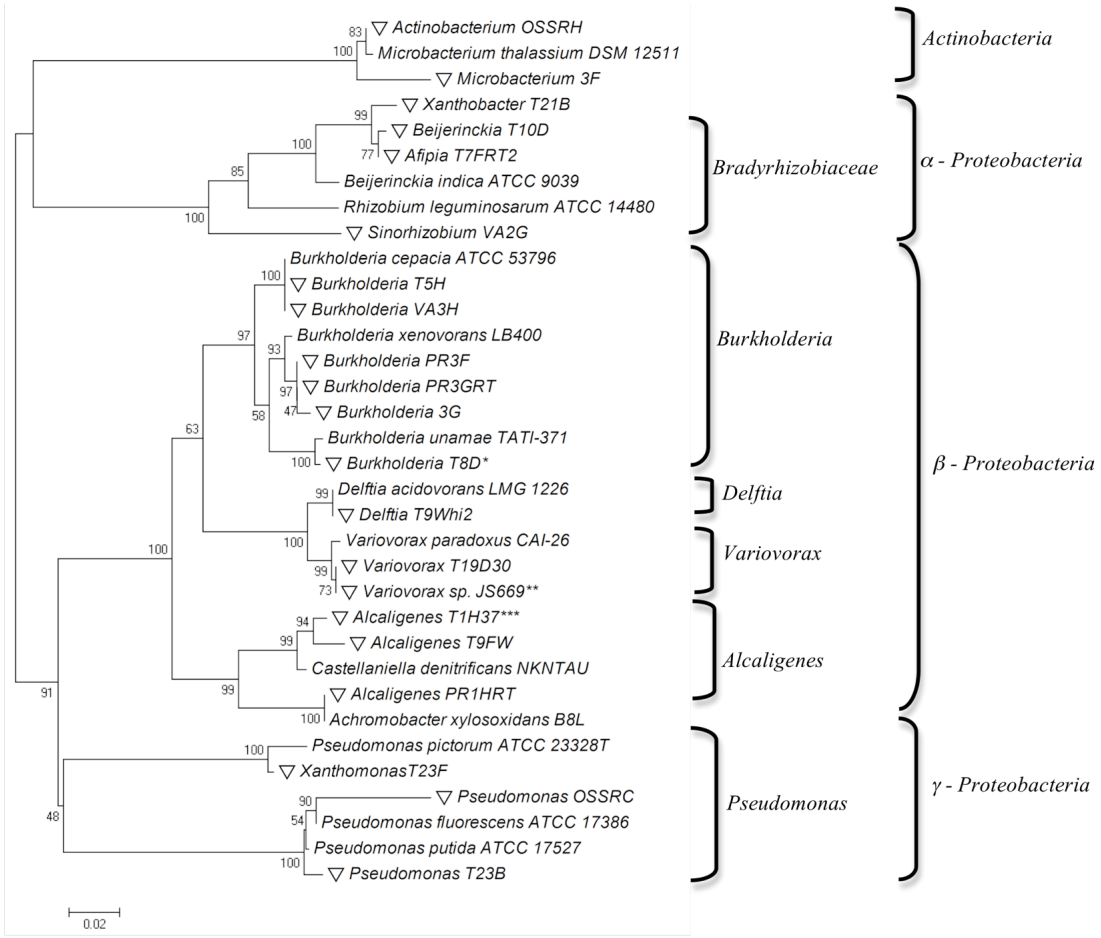
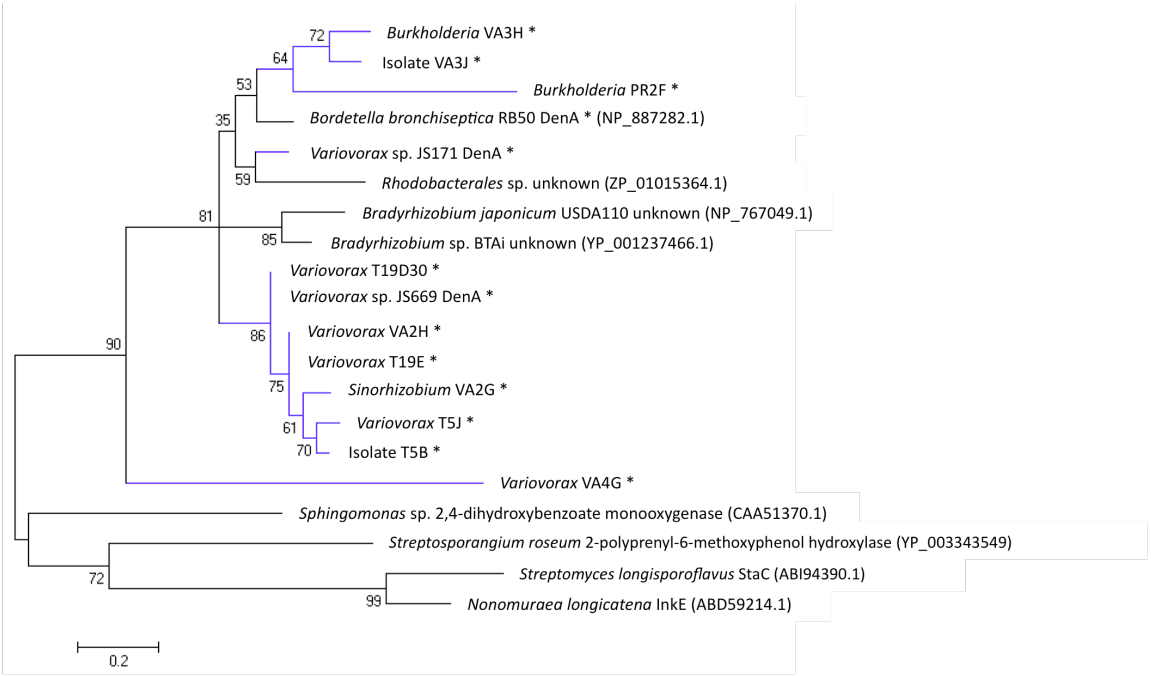


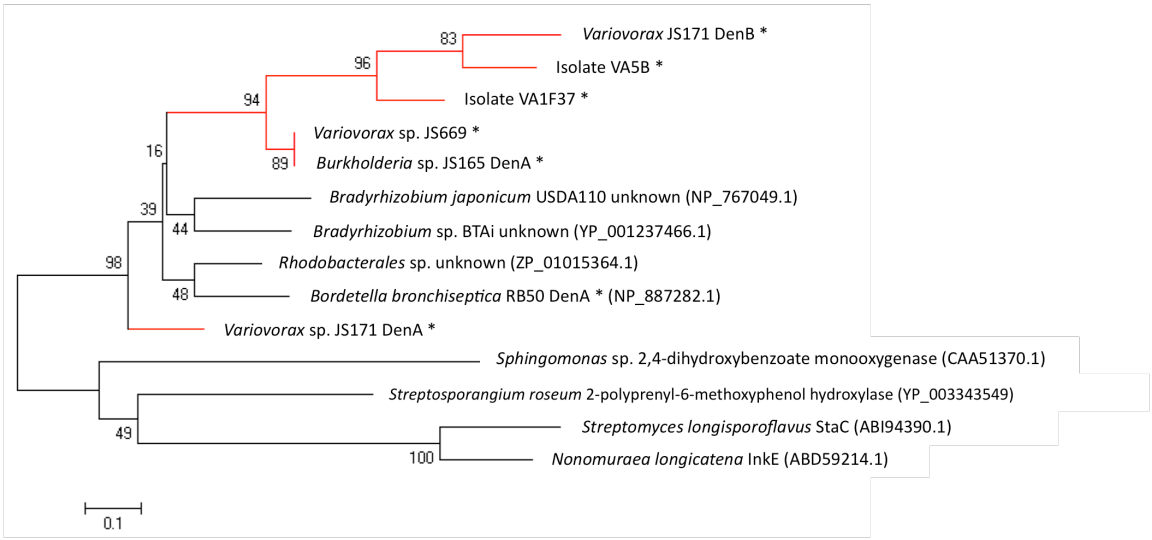
Figure 3.3. Phylogeny of 3NTyr-degrading isolates based on 16S rRNA sequences. A multiple sequence alignment was constructed with 16S rRNA sequences from 45 3NTyr-degrading isolates and their closest phylogenetic neighbors. Representative isolates were selected in three instances to represent large groups of isolates with >97% identity. Scale is in nucleotide substitutions per site.

Burkholderia* T8D represents sixteen isolates from ten sites. ***Variovorax* sp. JS669 represents six isolates from six sites. * *Alcaligenes* T1H37 represents five isolates from 4 sites.

3.4.3 Characterization of *denA* in 3NTyr-degrading isolates. To determine the diversity of DenA among the 3NTyr-degrading isolates we identified seventeen partial *denA* genes using a combination of degenerate primers (Figure 3.4). Initially, we used degenerate primers (DenGenF/DenGenR4) that were designed based on putative FAD monooxygenases that exhibited high identity with a putative *denA* from *Variovorax* sp. JS171 (19). Multiple sequence alignment with *denA* from the isolates obtained in the current study provided several well-conserved regions within the *denA* sequence (Fig. 2). Primers designed from the conserved regions identified in the multiple sequence alignment are not degenerate and are therefore highly specific probes for *denA* (502F/688R and 502F/849R). DenGenF/R4 amplification identified FAD monooxygenase *denA* sequences in less than 20% of isolates tested. 502F coupled with either 688R or 849R amplified *denA* sequences in closer to 50% of the isolates tested. Additionally, the combination of primers 502F, 688R and 849R identified a second putative *denA* in *Variovorax* sp. JS171 that we designated *denB*. The *denA* sequences group within a single clade of FAD monooxygenases that includes biochemically characterized DenA (Chapter 2) (Figure 3.4). Specifically, primer sets 502F/688R and 502F/849R in part target a region, 186 bp and 347 bp, respectively, of the *denA* that includes the FAD/NAD(P)H dual binding site, which is well conserved among FAD monooxygenases (11). Thus, the non-degenerate primer sets are specific probes for *denA* and enabled the discovery of *denA* in organisms that had previously not revealed *denA* with the DenGenR/R4 primers.


72
64
53
35
59
81
85
86
75
61
70
90
72
99
Burkholderia VA3H *
Isolate VA3J *
Burkholderia PR2F *
Bordetella bronchiseptica RB50 DenA * (NP_887282.1)
Variovorax sp. JS171 DenA *
Rhodobacterales sp. unknown (ZP_01015364.1)
Bradyrhizobium japonicum USDA110 unknown (NP_767049.1)
Bradyrhizobium sp. BTAi unknown (YP_001237466.1)
Variovorax T19D30 *
Variovorax sp. JS669 DenA *
Variovorax VA2H *
Variovorax T19E *
Sinorhizobium VA2G *
Variovorax T5J *
Isolate T5B *
Variovorax VA4G *
Sphingomonas sp. 2,4-dihydroxybenzoate monooxygenase (CAA51370.1)
Streptosporangium roseum 2-polyprenyl-6-methoxyphenol hydroxylase (YP_003343549)
Streptomyces longisporoflavus StaC (ABI94390.1)
Nonomuraea longicatena InkE (ABD59214.1)
0.2

(A)


83
96
94
16
89
39
44
98
48
49
100
Variovorax JS171 DenB *
Isolate VA5B *
Isolate VA1F37 *
Variovorax sp. JS669 *
Burkholderia sp. JS165 DenA *
Bradyrhizobium japonicum USDA110 unknown (NP_767049.1)
Bradyrhizobium sp. BTAi unknown (YP_001237466.1)
Rhodobacterales sp. unknown (ZP_01015364.1)
Bordetella bronchiseptica RB50 DenA * (NP_887282.1)
Variovorax sp. JS171 DenA *
Sphingomonas sp. 2,4-dihydroxybenzoate monooxygenase (CAA51370.1)
Streptosporangium roseum 2-polyprenyl-6-methoxyphenol hydroxylase (YP_003343549)
Streptomyces longisporoflavus StaC (ABI94390.1)
Nonomuraea longicatena InkE (ABD59214.1)
0.1

(B)

Figure 3.4. Phylogeny of HNPA denitrases among 3NTyr-degrading isolates. Phylogeny of HNPA denitrases from 3NTyr-degrading isolates was determined using amplicons from degenerate primers (A) Primer set DegenF/R4 (19) and (B) Primer set 502F, 688R, and 849R. A multiple sequence alignment was constructed of the partial DenA sequences from isolates along with their closest phylogenetic neighbors. The results were used to construct a Neighbor-joining phylogenetic tree using MEGA4 (25). The percentages of replicate trees in which the associated taxa clustered in the bootstrap test (500 replicates) are shown next to the branches of the tree (12). The evolutionary distances were computed using the Poisson correction method (31) and are in the units of the number of amino acid substitutions per site. The branches representing HNPA denitrases in isolates with biochemically characterized HNPA denitrase activity are indicated (*).

3.4.4 Context for related *denA*. The gene context for *denA* was examined in several 3NTyr-degrading isolates. DenA activity was detected in clones of *denA* from *Bordetella bronchiseptica* RB50 and *Variovorax* sp. JS171 (Chapter 2, (19)), however 3NTyr metabolism was only detected in JS171 and not RB50. The context of *denA* and *denA*-like genes from *Bordetella bronchiseptica* RB50, *Bradyrhizobium japonicum* USDA110, *Bradyrhizobium* sp. BTAi and *Rhodobacterales* bacterium HTCC2654 were compared, along with JS669, JS165 and JS171 (18). Genes near *denA* are not conserved among the compared genomes, with the exception of the presence of a LysR-family transcriptional regulator upstream of the *denA* and related genes that is present in all of the sequences except for *B. bronchiseptica* RB50. Only *B. bronchiseptica* RB50 contains the genes for

homoprotocatechuate degradation directly downstream of the *denA* sequence within the same operon. There are homoprotocatechuate degradation genes in the two *Bradyrhizobium* strains as well as the *Rhodobacterales*, however they are not directly associated with *denA*. The results indicate that the organization of genes downstream of *denA* in JS171 is most similar to that of *B. bronchiseptica* RB50.

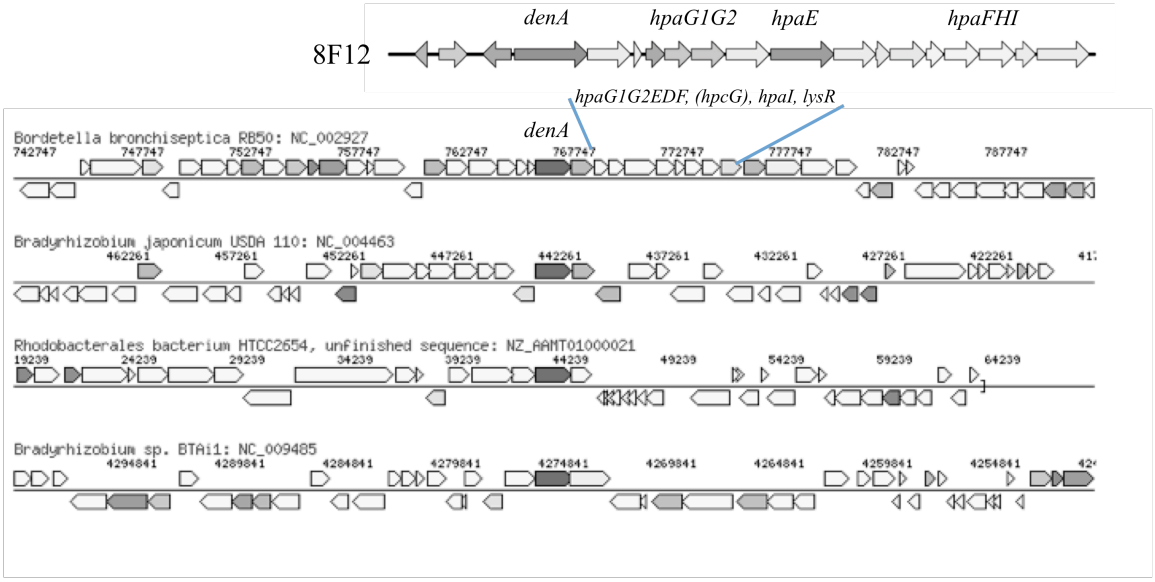


Figure 3.5. Gene neighborhoods surrounding *denA* and putative *denA*. *denA* are labeled (charcoal), putative *denA* are colored (charcoal) and genes that encode enzymes for HPC degradation are labeled.

3.5 DISCUSSION

The facile isolation of over one hundred 3NTyr-degrading microbes implies the widespread presence of 3NTyr or its analogs in the habitats occupied by the bacterial isolates. The results support the importance of 3NTyr in the sampled ecosystems.

The majority of the 3NTyr-degrading isolates release nitrite from HNPA, implying that the currently known 3NTyr biodegradation pathway may be universal (18). The presence of similar *denA* sequences in half of the tested 3NTyr-degrading isolates indicates that the gene encoding the HNPA denitrase is conserved among such isolates. The inability of the *denA* probes to identify related sequences in the other isolates indicates the need to further characterize such isolates in order to identify either divergent genes encoding HNPA denitrases or a different mechanism for 3NTyr metabolism. Furthermore, the presence of two putative HNPA denitrases (DenA and DenB) in JS171 is intriguing and requires further investigation. An HNPA denitrase from JS171 has been biochemically characterized (19) but the gene encoding DenA is not transcriptionally upregulated in 3NTyr-grown cultures in spite of the fact that the 3NTyr degradation pathway is inducible (data not shown). Therefore, it is plausible that DenB is responsible for the denitration of HNPA during the course of 3NTyr degradation, whereas DenA is a separate enzyme with denitrase activity. The biochemical roles of both DenA and DenB in JS171 are currently under investigation.

The widespread capability for 3NTyr degradation among sites examined in the current study implies its importance in multiple ecological settings, however the niche of the 3NTyr degraders is unknown. The biosynthesis of 3NTyr by diverse animals (5, 10,

16) and plants (8, 21) implies widespread bioavailability of the compound. 3NTyr degradation could play any of several ecological roles. **(1)** Heterotrophic soil microbes could encounter 3NTyr excreted by plants and animals and subsequently metabolize the nitro compound as a growth substrate. **(2)** 3NTyr degradation might provide a defense mechanism to protect the microbes from 3NTyr excreted by plants or animals, or from endogenous protein nitration. **(3)** 3NTyr can be formed by the action of atmospheric reactive nitrogen species on airborne protein (pollen) and the 3NTyr degraders may be using such proteins as a growth substrate (13). **(4)** During infection and inflammation there is a marked increase in nitric oxide synthase activity and reactive nitrogen species formation in macrophages and haemocytes. Hence there is also the potential for host-microbe interactions related to the host producing 3NTyr during infection (5, 8, 10, 14, 16) and bacteria subsequently metabolizing the 3NTyr. Future research will explore these hypotheses to identify the ecological role of 3NTyr degradation.

ACKNOWLEDGEMENTS

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CHAPTER 4

DETERMINATION OF THE BIODEGRADATION PATHWAY OF 2-NITROPROPYLBENZENE AND ITS NATURAL ANALOG 1-NITRO-2-PHENYLETHANE IN *BURKHOLDERIA* SP. JS670

4.1 ABSTRACT

Bacterial growth on nitroaliphatic compounds has previously not been reported. Several nitroaliphatic compounds are naturally produced, and potentially are biodegradable by indigenous bacteria. 1-Nitro-2-phenylethane (NPE) is an aromatic compound with a nitroaliphatic substituent produced by a variety of plants. Using a combination of molecular and biochemical methods, we identified the biodegradation pathway for NPE and a less toxic analog, 2-nitropropylbenzene (NPB) in *Burkholderia* sp. JS670. NPE degradation results in the accumulation of 3-nitropropionic acid, a potent toxin, which has not previously been identified as a metabolite from bacteria. Additionally, the genes that encode required enzymes for NPE/NPB degradation, *npeA1* and *npeD*, are similar to those genes that encode the biphenyl dioxygenase alpha subunits and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolases that are involved in biphenyl degradation. Oxygen uptake experiments with JS670 demonstrated that the NPE/NPB pathway is inducible, and both *npeA1* and *npeD* are transcriptionally upregulated in cultures grown on NPB. An *npeA1* mutant failed to grown on NPE/NPB but maintained

the ability to degrade biphenyl, indicating that there is a separate dioxygenase alpha subunit for biphenyl. NpeA1 catalyzed the transformation of NPE and NPB as well as several structural analogs, including phenylethylamine, nitrostyrene and trans-β-nitrostyrene. Both NpeA1 and NpeD represent novel branches within their phylogenetic groups. The results presented here support the hypothesis that the biodegradation pathway for NPB is used for a variety of substituted aromatic compounds by eliminating the side chain and using the remaining compound as a carbon and energy source.

4.2 INTRODUCTION

Previously considered to be rare, natural nitro compounds have been discovered in a growing number of ecosystems. The absence of accumulation of such compounds in the environment suggests the ubiquitous presence of pathways for their degradation. The pathways of biosynthesis and degradation, the flux and the ecological roles of most natural nitro compounds are unknown and represent a substantial unexplained source of metabolic diversity (59). In contrast, a considerable body of literature is available on the degradation of anthropogenic nitro compounds owing to the toxicity and persistence of such compounds in polluted ecosystems (36, 61).

Microorganisms employ a variety of mechanisms to degrade anthropogenic nitro compounds, and the pathways appear to have evolved relatively quickly following the introduction of such compounds into the biosphere (18, 19, 61). Rapid evolution of catabolic pathways for such compounds implies that there are preexisting pathways that

serve as sources of metabolic diversity. Recruitment of enzymes from metabolic pathways for natural compounds is a likely contributory source for anthropogenic compound degradation pathways. Study of the degradation pathways for natural nitro compounds will help to clarify hypotheses about the evolution of catabolic pathways for anthropogenic compounds.

The genes that encode key enzymes for biphenyl and toluene biodegradation have been identified (6, 17, 23, 26, 28, 39). Oxygenases activate the aromatic ring by incorporation of molecular oxygen into the structure being metabolized (55). Oxygenases, such as those involved with toluene and biphenyl degradation, tend to have a broad substrate specificity (36, 58).

1-Nitro-2-phenylethane (NPE) is a flavor-related, volatile nitro compound produced by an assortment of higher plants including tomatoes (54), rosewood trees (21), sassafras, jasmine (41), Wright's gardenia and orchids (2, 29). NPE is a secondary plant metabolite formed along with other aromatic and aliphatic compounds (54) that appear to be involved with pollination, either as a scent used to attract insects for pollination, or as pesticide-related compounds. It is possible that NPE serves to repel insect predators, as do other similarly produced compounds such as 2-phenylethanol and glucosinolates (34, 54). 2-Nitropropylbenzene (NPB) is a synthetic analog of NPE, with an extra methyl group on the aliphatic substituent.

Biosynthesis and biodegradation control the flux of NPE in natural ecosystems. Nothing is known about the degradation or flux of NPE in natural ecosystems and there is only limited research on the ecological impact of NPE (2, 34). We hypothesized that microbes capable of degrading NPB might also be able to degrade NPE. We report here

the isolation of bacteria capable of biodegradation of NPE/NPB via an oxidative catabolic pathway.

4.3 MATERIALS AND METHODS

4.3.1 2-Nitropropylbenzene synthesis. NPB was synthesized by reduction of *trans*-β-methyl-nitrostyrene with sodium borohydride (57). NPB identity was confirmed by GC-MS and comparison to published data (9) (Scifinder, American Chemical Society).

4.3.2 Isolation and growth of bacteria. Soil from beneath tomato plants in Dacula, GA was suspended (10%) in minimal media (BLK-N, pH 7.2) containing NPB (0.5 mM) (8). Cultures were grown with shaking at 30^{o}C. Following several transfers into fresh media, serial dilutions were made of the enriched culture and spread onto BLK-N plates (1.5% agar) supplemented with NPB or NPE (1 mM) as the carbon source. Individual colonies that grew on the NPB selective plates caused yellow discoloration of the media in the plates. The colonies were purified and archived for further study. Liquid cultures of NPB-degrading isolates were routinely maintained in BLK-N supplemented with NPB or NPE (2 mM) or ¼ strength tryptic soy broth (TSB).

4.3.3 Analytical methods. NPB and NPE were analyzed by HPLC using methods previously described (38). 3-Nitropropionic acid (3NPA) was analyzed with an a isocratic mobile phase (75% 1.74 mM H_2SO4 in H_20, 25% acetonitrile) at a flow rate of

0.3 ml/min on an IC Sep ICE-ION-310 Fast column (6.5 x 150 mM), using a Varian HPLC system equipped with photodiode array detector at a wavelength of 207 nM. Protein was measured using a Pierce BCA protein assay reagent kit (Rockford, IL).

4.3.4 Indole assay. Cultures were grown at 30^{o}C on BLK-N plates (1.5% agar) supplemented with NPB (2 mM) or on ¼ strength TSB plates (1.5% agar). After 12-24 hours of growth, indole crystals were placed in the lid of the agar plates. The indole-containing plates were sealed with parafilm and allowed to continue growing at 30^{o}C for another 24-48 hours. Colonies with the ability to convert indole to indigo turned blue (14, 31).

4.3.5 Whole cell assay. NPB-induced cells were grown to log phase, harvested by centrifugation (2566 x g, 4^{o}C, 10 minutes) and then washed once in phosphate buffer (25 mM, pH 7). Cells were suspended in 1/5 volume air-saturated phosphate buffer with the addition of NPE (2 mM) and NH_4Cl (9.4 mM). Duplicate samples (300 μl) were periodically removed, mixed with 50:50 acetonitrile:water (300 μl) and analyzed by HPLC as described above.

4.3.6 Respirometry. To measure oxygen uptake by resting cells, cultures were grown in BLK-N with NPB (10 mM), biphenyl (0.2%), or succinate (7.5 mM) as the carbon source. Cells in log phase were harvested by centrifugation, washed twice with phosphate buffer (25 mM; pH 7.0), and suspended to an OD_{600} of 1.5 in air-saturated phosphate buffer. Oxygen uptake was measured polarographically with a Clark-type oxygen

electrode connected to a YSI model 5300 biological oxygen monitor (25^{o}C). All substrates were dissolved in N, N-dimethylformamide (DMF), except for succinate, which was dissolved in water.

4.3.7 DNA and RNA extraction. DNA was extracted from individual colonies using the Promega SV Wizard Genomic DNA purification kit (Madison, WI.) For RNA extraction, cells were harvested during log phase by centrifugation. Cell pellets were stored in 0.5 ml RNA Later (Applied Biosystems, Foster City, CA). RNA was extracted using the Promega SV Wizard Total RNA Isolation kit according to the manufacturer's instructions (Madison, WI). RNA was routinely checked for DNA contamination via 16S rRNA PCR as described below. When necessary, RNA samples were incubated at 37^{o}C for 55 minutes with DNase I (Fermentas, Glen Burnie, MD) and subsequently incubated with 1U DNase Stop Solution (Promega, Madison, WI) at 65^{o}C for 10 minutes. Reverse transcription of the purified RNA was subsequently performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

4.3.8 PCR conditions, primer design and DNA sequencing. PCR amplification of the partial 16S rRNA gene was conducted using 341F and 926R (5) (Table 4.1). PCR reactions with other primer sets were similar to those used for 16S rRNA amplification except for noted changes (Table 4.1). Amplicons were purified using Wizard SV Gel and PCR Clean up Systems (Promega, Madison, WI). Purified amplicons were sequenced by the Nevada Genomics Center (Reno, NV). Degenerate primers were designed using

sequences from organisms that were previously used to make degenerate hydrolase primers (26) as well as the sequence encoding the 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase from *Burkholderia cepacia* LB400 (GenBank, Accession number YP 556398). The degenerate hydrolase primers amplify a 342 bp region of the gene encoding the hydrolase, specifically targeting conserved regions surrounding the catalytic nucleophile. All primers designed in this study were synthesized by Integrated DNA Technologies (Coralville, IA).

Primer	Sequence	Ta[1] (^oC)	Time[a] (sec)	Time[e] (sec)	[$MgCl_2$] (mM)	Source
degenhydF4	5'-TGGAGYAACTAYTACCGCAA	50	60	90	1.5	This study
degenhydR3	5'- TTGAACAGCAGCTTGATGCC	50	60	90	1.5	This study
341F	5'- CCTACGGGIGGCIGCA	50	30	60	3.125	(5)
926R	5'- CCGICIATTIITTTIAGTTT	50	30	60	3.125	(5)
BPH 1F	5'- GGACGTGATGCTCGAYCGC	57	60	60	2	(6)
BPH 1R	5'-TGTTSGGYACGTTMAGGCCCAT	57	60	60	2	(6)
TodC1F	5'-CAGTGCCGCCAYCGTGGYATG	66	60	120	1.5	(23)
TodC1R	5'-GCCACTTCCATGYCCRCCCCA	66	60	120	1.5	(23)
KONPEKT ODC1F	5'-CTCGAATTCTACATGTCGCTGC AGAACTG	55	60	120	1.5	This study
KONPEKT ODC1R	5'-CTCGTCGACAATGTGCCCTTCG AGAAGGA	55	60	120	1.5	This study
LacZR	5'-CGCCAAGACTGTTACCCATC	54.4	60	120	2.5	(27)

[1] Annealing Temperature, [a] Annealing Time, [e] Extension Time.

Table 4.1. Primers and PCR conditions. PCR reactions (20 μl) contained template DNA along with dNTPs (400 μM), forward and reverse primers, $MgCl_2$ (1 μM), 1X GoTaq Flexi Buffer and 1U Go Taq DNA Polymerase (Promega, Madison, WI). All PCR began with a preincubation at 95^{o}C (5 min) followed by 35 cycles of denaturation at 95^{o}C

(2 min), primer annealing and DNA extension at 72°C. The final PCR cycle ended with an extension at 72°C (10 min).

qRT-PCR thermocycling for measurement of *npeA1* and 16S began with two minutes at 50°C (Stage 1), followed by ten minutes at 95°C (Stage 2). After the first two temperature cycles there were forty repetitions for Stage 3 of Step 1-95°C (15 sec), Step 2-57°C (60 sec), and Step 3-72°C (60 sec). Data collection occurred during Stage 3, Step 1. A final dissociation stage included 95°C (15 sec), 60°C (60 sec) and 95°C (15 sec). qRT-PCR conditions for *npeD* were identical to those described above, except for Stage 3, Step 2 which was done at 55°C (60 sec).

Degenerate nucleotide bases were used to increase the range of primer detection: Inosine (I) pairs with adenine (A), cytosine (C) or uracil (U); (Y) pairs with C or thymine (T); (M) pairs with A or C, (S) pairs with G or C; and (R) pairs with A or G.

4.3.9 Alignments and phylogenetic trees. 16S rRNA gene sequences of NPE-degrading isolates were examined via BLASTN analysis (NCBI) (http://www.ncbi.nlm.nih.gov) (3). Highly related sequences of NpeA1 (dioxygenase) or NpeD (hydrolase) were identified via BLASTP analysis (NCBI) (3). Protein families and conserved regions were identified using the Pfam (http://pfam.sanger.ac.uk/) and CDD (35) databases (7, 51, 52). Nucleotide and amino acid sequences for comparison were obtained from NCBI. Alignments were conducted in Bioedit using Clustal W (EBI, (53)) . Phylogenetic trees were constructed with MEGA4 using the neighbor joining method and bootstrap analysis (500 replicates) (15, 45, 49). Evolutionary distances for proteins were computed using the Poisson correction method (62). Evolutionary distance for nucleic acids were

computed using the Maximum Composite Likelihood Method (50). Redundant and hypothetical results were omitted prior to final tree construction.

4.3.10 Quantitative reverse transcription PCR (qRT-PCR). qRT-PCR was performed on an Applied Biosystems 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA). Gene copy calculations were done as previously described (44). Standard curves were constructed using ten-fold serial dilutions of the target fragment. Triplicate cDNA samples were assayed for transcription of each target gene. qRT-PCR reactions (30 μl) contained forward and reverse primers (KONPEKTODC1F/R, NPEKHydrolase F/R or NPEK 16SF/R (0.2 μM each), (Table 4.1) and 1X SYBR Green PCR Master Mix with a balance of Nuclease-free H_2O (Ambion, Applied Biosystems, Foster City, CA).

4.3.11 Insertional mutagenesis of *npeA1* in *Burkholderia* sp. JS670. A partial fragment of *npeA1* (311 bp) from *Burkholderia* sp. JS670 was amplified using primers KONPEKTODC1F/R (Table 4.1). The *npeA1* fragment was cloned into pVIK110 using EcoRI (1U) and SalI restriction sites (1U) (New England Biolabs, Ipswich, MA) (30). The resulting *npeA1*:pVIK110 vector construct was then electroporated into electrocompetent *E. coli* S17-1 λ-pir cells (30) (2.5 kV, 25 μF, 200 Ω). Electroporated cells were briefly recovered in l ml SOC media and then plated onto Luria-Bertani Broth (LB) (1.5% agar) supplemented with kanamycin (30 μg/ml) (30, 46). *npeA1*:pVIK110 was purified from *E. coli* S17-1 λ-pir cells using the Wizard SV Miniprep kit (Promega, Madison, WI) according to manufacturer's instructions. *npeA1*:pVIK110 (200ng) was

subsequently electroporated into *Burkholderia* sp. JS670 (100 µl) using the settings described above. Electrocompetent *Burkholderia* sp. JS670 were prepared as previously described (11). Transformants were recovered in ¼ strength TSB. Correct insertion of the suicide vector was confirmed via PCR using a combination of primers TODC1F/R, KONPEKTODC1F/R and LacZR (27) for amplification and sequencing through Nevada Genomics.

4.3.12 Auxanography. Auxanography to assay growth on a variety of carbon sources with *Burkholderia* sp. JS670 and JS670 *npeA1*::pVIK110 was employed as previously described (22). Potential carbon sources were chosen based on both structure and relatedness to secondary plant metabolites.

4.3.13 Chemicals. 1-Nitro-2-phenylethane was from Apin Chemicals (Abingdon, Oxon, UK). Benzoic acid, benzyl alcohol, indole, sodium borohydride, toluene, biphenyl, 3-nitropropionic acid and trans-β-methyl-nitrostyrene were from Sigma-Aldrich (Milwaukee, WI). Trans-β-nitrostyrene was from Alfa Aesar (Ward Hill, MA). Trans-cinnamic acid and phenylethylamine, were from Acros Organics (Geel, Belgium). NPB was synthesized as previously described (57).

4.4 RESULTS

4.4.1 Isolation and identification of *Burkholderia* sp. JS670. Ten isolates were obtained by selective enrichment with NPB as the carbon source. Concomitant with growth, isolates routinely removed all NPB from the culture medium (Figure 4.1). The 16s rRNA gene of one isolate had 99% similarity to that of *B. cepacia* [Accession number FJ169472] and was designated *Burkholderia* sp. JS670.

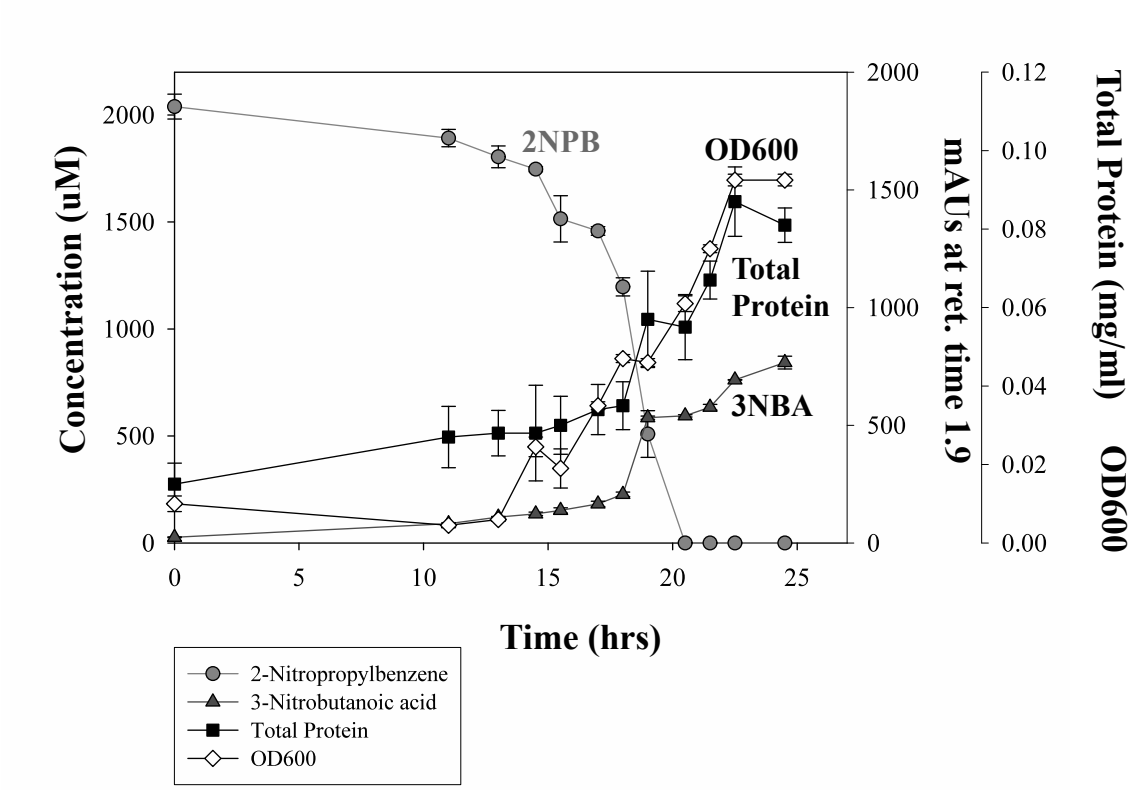


Figure 4.1. Growth of *Burkholderia* sp. JS670 on 2-nitropropylbenzene.

4.4.2 Culture observations. During growth on NPB or NPE, *Burkholderia* sp. JS670 produced a yellow metabolite in the culture media that disappeared upon acidification. The UV spectrum of the yellow metabolite was similar to previously reported UV spectra of *meta*-cleavage products (37). Additionally, 3-nitrobutanoic acid (3NBA) or 3NPA accumulated during growth on NPB or NPE at approximately 40% of the stoichiometric amount. These results indicate that some of the intermediates are being metabolized or otherwise transformed. Conversely, it is possible that not all of the NPE/NPB is being used as a growth substrate. When grown on NPB, but not on ¼ TSB, *Burkholderia* sp. JS670 transformed indole into indigo as evidenced by the accumulation of a blue color within the colonies, thus indicating that dioxygenase activity was involved in growth on NPB (33, 56). Interestingly, *Burkholderia* sp. JS670 grown on NPE/NPB readily transformed biphenyl and biphenyl grown JS670 readily transformed NPE/NPB. The preliminary observations thus implied that the metabolic pathways for NPE/NPB and biphenyl were similar.

4.4.3 Respirometry. To determine both utilization of oxygen during NPB degradation and inducibility of the NPE/NPB degradation pathway, oxygen uptake was measured in whole cells. Stimulation of oxygen uptake by NPB and biphenyl in NPB- and biphenyl-grown cells, but not in succinate-grown cells, indicated that the enzymes catalyzing degradation of both NPB and biphenyl were inducible (Table 4.2). Additionally, based on the cross-induction in NPB- and biphenyl-grown cells it appeared that the pathways for degradation of the two compounds were related. Lack of stimulation of oxygen uptake by toluene indicated that the NPE/NPB pathway is not inducible by toluene.

Substrate (100 μM)	Biphenyl-grown JS670[1, 2]	NPB-grown JS670[1]	Succinate-grown JS670[1, 2]
Biphenyl	**313**	**474**[4]	**54**
NPB	**163**	**339**[4]	**28**
Toluene	**68**	**90**[2]	**17**
Succinate	**27**	**38**[3]	**38**
Dimethylformamide (solvent)	**0**	**34**[2]	**6**

[1]Average nmol O_2 consumed per min per mg protein, [2]Average of results from duplicate samples, [3]Average of results from triplicate samples, [4]Average of results from quadruplicate samples, typical results presented.

Table 4.2. Respirometry with *Burkholderia* sp. JS670.

4.4.4 NPE transformation by NPB-grown *Burkholderia* sp. JS670. Cultures were routinely grown on NPB, rather than NPE, because it was less toxic to the cells. NPB-grown *Burkholderia* sp. JS670 resting cells readily degraded NPE with no lag (Figure 4.2). During growth on NPE there was a 0.081 mg/ml increase in total protein, and a growth yield of 30 mg of protein/ mmole NPE consumed, which suggests that *Burkholderia* sp. JS670 might only use a portion of the molecule for carbon and energy. The growth yield is similar, though slightly less than yields reported from *Burkholderia* sp. JS667 growing on diphenylamine (48) and *Burkholderia xenovorans* LB400 growing on biphenyl (43). Appearance of 3-nitropropionic acid was concomitant with NPE

disappearance. The lack of lag time, ready degradation of NPE and release of the side chain into the media suggest that the NPB and NPE degradation pathways are the same.

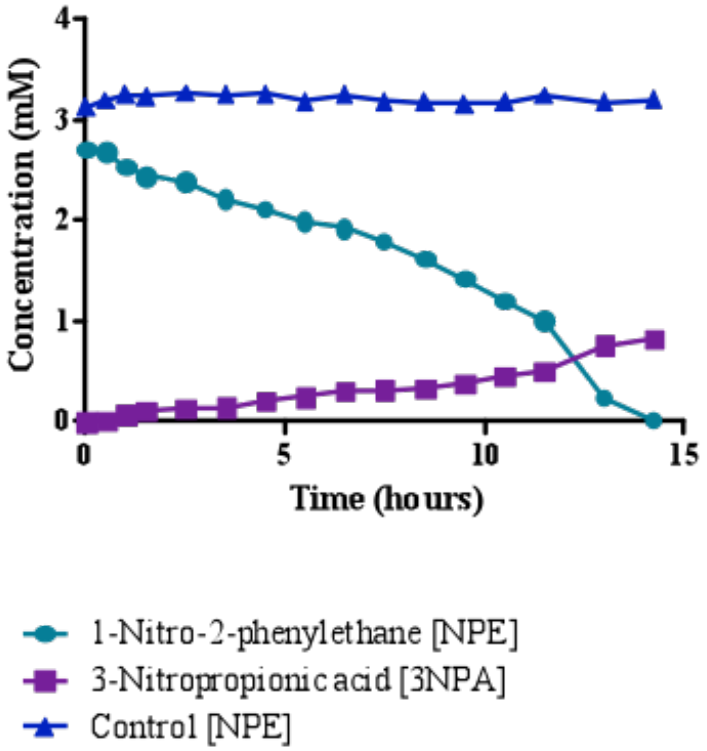
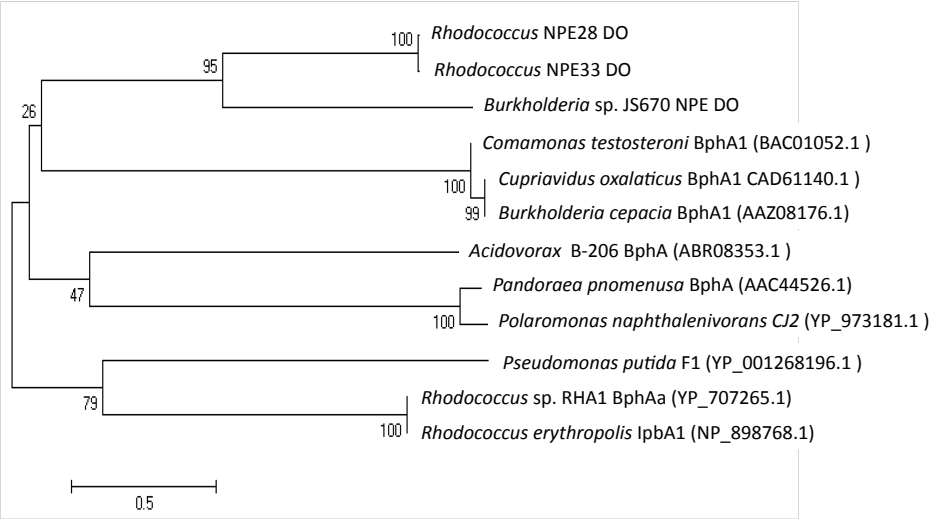


Figure 4.2. Transformation of NPE and concurrent accumulation of 3NPA by *Burkholderia* sp. JS670. *Burkholderia* sp. JS670 demonstrated a 0.08 mg/ml increase of protein concentration. The concentration of 3NPA produced is approximately 40% of the concentration of NPE consumed.

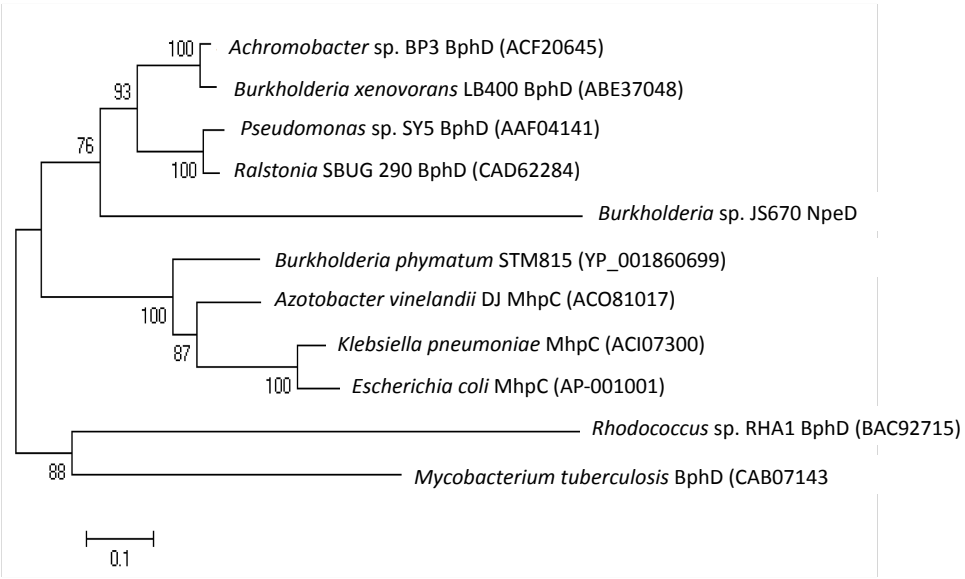
4.4.5 Identification of *npeA1* and *npeD*. To identify genes required for NPE/NPB degradation, previously described degenerate primers were used to amplify and identify the dioxygenase that might catalyze the first step of the pathway (23). Based on the proposed NPE/NPB pathway, a second gene encoding a hydrolase was targeted for

identification. Degenerate primers were designed based on a multiple sequence alignment of *Pseudomonas pseudoalcaligenes* KF707, *Pseudomonas* sp. KKS102, *P. putida* sp. KF715, and *Burkholderia cepacia* LB400 and used to identify the hydrolase (342 bp) involved with NPE/NPB degradation. TBLASTX analysis of sequenced amplicons revealed enzymes that were closely related to the fragments (3). The amplicon obtained from *Burkholderia* sp. JS670 using degenerate dioxygenase primers shares 77% identity with the *Cupriavidus necator* BphA1 biphenyl dioxygenase (Accession number CAA66598) and was named *npeA1*. NpeA1is similar to other Rieske non-heme iron dioxygenases, including several biphenyl dioxygenases. As with other members of the enzyme family, the alpha subunit contains an N-terminal Rieske domain [2Fe-2S] and a C-terminal catalytic domain with a mononuclear Fe (II) binding site (19, 60).

The translated amplicon obtained using degenerate hydrolase primers shares 85% identity with that of the *Burkholderia xenovorans* LB400 BphD 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (Accession number YP 556398.1). Thus, the gene identified was named *npeD* and encodes a hydrolase involved with NPE/NPB degradation. The amplified region of the NPE/NPB hydrolase includes several conserved motifs surrounding the putative nucleophilic serine residue (13, 16, 32). Nine residues upstream from the nucleophile is the DALGI motif, and further upstream from the DALGI motif is a secondary consensus sequence including RVI. Thirteen residues downstream from the nucleophilic serine is the PERL sequence, which is highly similar to the PERV consensus sequence. Both NpeA1 and NpeD are sufficiently distinct from biphenyl homologues and thus form deeply branched groups of the dioxygenase and hydrolase phylogenetic trees (Figure 4.3).



(A)



(B)

Figure 4.3. Phylogenetic trees of NpeA1 (A) and NpeD (B). Multiple sequence alignments were constructed using the top BLASTP matches to NpeA1 and NpeD in Bioedit. The results were used to construct neighbor-joining phylogenetic trees using MEGA4 (49). The percentage of replicate trees in which the associated taxa clustered

together in the bootstrap test (500 replicates) are shown adjacent to the branches (15). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (62) and are in the units of the number of amino acid substitutions per site.

4.4.6 Transcription of *npeA1* and *npeD*. To determine whether transcription of *npeA1* and *npeD* were inducible, qRT-PCR analyses with both genes were conducted and compared to 16S rRNA gene transcription. Efficiency for all qRT-PCR reactions was greater than 87.9% (slope between -3.33 and -3.65) and R^2 values for all qRT-PCR reactions was greater than 0.98. NPB-grown JS670 demonstrated a 74-fold increase in transcription of *npeA1* compared to succinate grown cells and a 53-fold increase in transcription as compared to biphenyl-grown cells. There was a 197-fold increase in transcription of *npeD* when cells were grown on NPB as compared to biphenyl and a 230-fold increase in transcription with NPB-grown cells compared to succinate-grown cells. Biphenyl-grown cells displayed less than a two-fold increase in transcription of both *npeA1* and *npeD* compared to succinate-grown cells. Transcription of *npeD* and *npeA1* were not upregulated in succinate-grown cells. Upregulation of *npeA1* and *npeD* is consistent with respirometry experiments that indicate the NPE/NPB degradation pathway is inducible and separate from biphenyl metabolism.

4.4.7 Loss of function mutant analysis. To demonstrate the role of NpeA1 in NPE and NPB metabolism, an insertional mutant in *Burkholderia* sp. JS670 *npeA1* was constructed

and tested. Although the mutant was unable to grow on NPE or NPB, growth on biphenyl was unaltered. The lack of growth of the mutant with NPE or NPB as the carbon source confirmed that *npeA1* is specifically required for metabolism of such compounds by JS670, but is not required for growth on biphenyl.

4.4.8 Auxanography with structurally related compounds. Growth experiments were conducted with a variety of substituted aromatic compounds. Auxanography with *Burkholderia* sp. JS670 and JS670 *npeA1*::pVIK110 revealed a pattern of growth consistent with the involvement of the NPE degradation pathway initial dioxygenase with several compounds. *Burkholderia* sp. JS670 readily grew on NPE, NPB, trans-β-nitrostyrene, trans-β-methyl-nitrostyrene and phenylethylamine, whereas the JS670 *npeA1*::pVIK110 mutant failed to grow on the same substrates, providing compelling evidence that the initial NPE/NPB dioxygenase is involved in the degradation of the NPE analogs. Biphenyl, benzoic acid and benzyl alcohol supported growth of both wildtype JS670 and the *npeA1* mutant, indicating that the NPE dioxygenase was not necessary for growth with such compounds. Cinnamic acid was not a growth substrate for JS670.

4.5 DISCUSSION

The pathway for NPE/NPB degradation in *Burkholderia* sp. JS670 was determined to be similar to that for biphenyl degradation (12), proceeding first through a dioxygenase-mediated upper pathway, and subsequently through a hydrolase-mediated

lower pathway. The isolate then releases the side chain from the parent compound (3NBA from NPB, 3NPA from NPE) and uses the remaining dienoate as a carbon source (Figure 4.4).

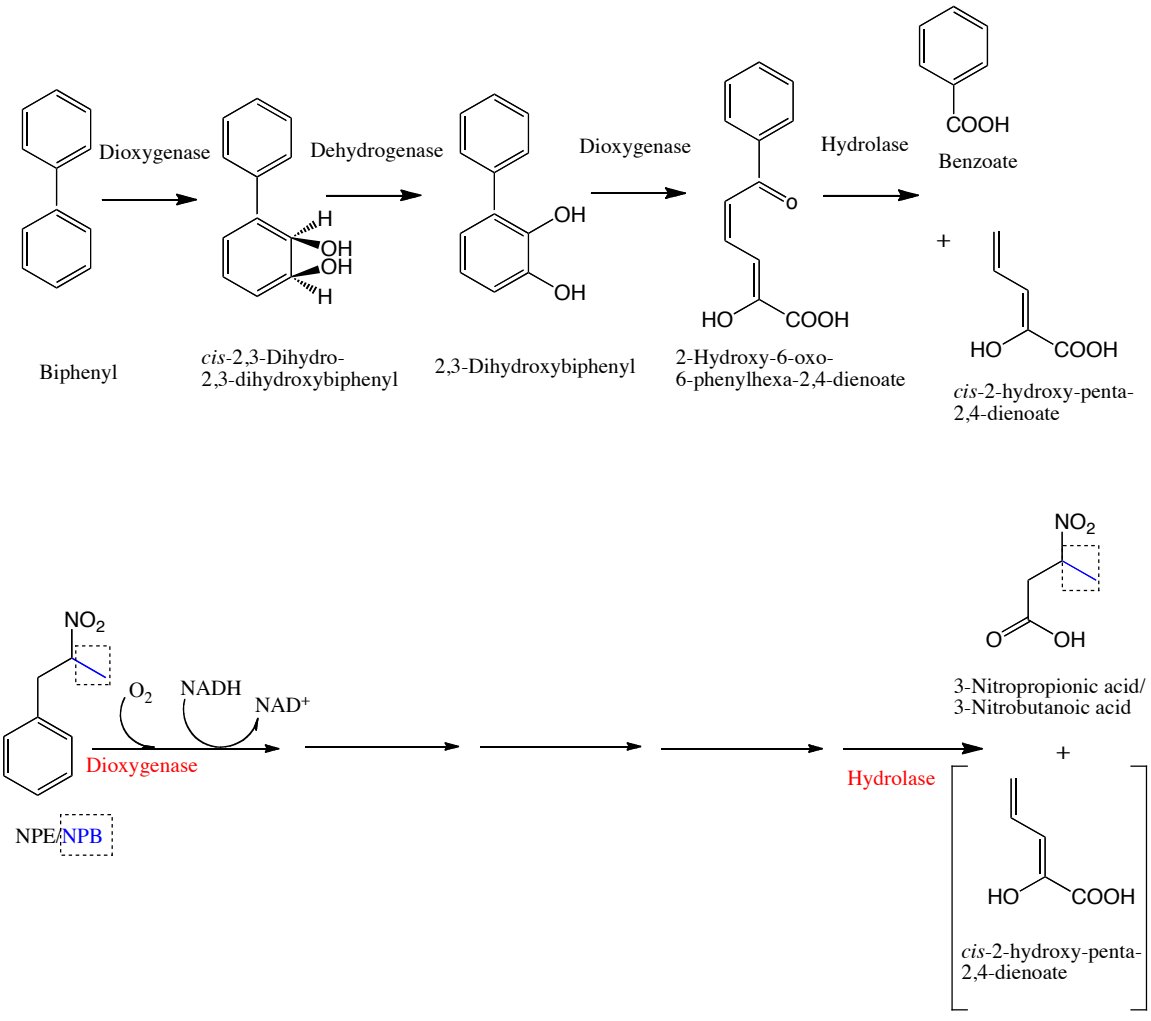


Figure 4.4 Biphenyl degradation pathway compared to proposed steps of the NPE/NPB biodegradation pathway. The NPB pathway is delineated from the NPE pathway ( ).

In accordance with oxygen uptake experiments, which demonstrated an inducible pathway for NPE/NPB degradation, qRT-PCR indicated significant up-regulation of *npeA1* and *npeD* in NPB-grown cultures. The significant increase in transcription with NPB-grown cultures as compared to biphenyl-grown cultures, indicates that *Burkholderia* sp. JS670 contains two separate catabolic pathways, one for NPB/NPE and one for biphenyl. The presence of two separate dioxygenases was confirmed via the mutant analysis.

Although both the biphenyl and NPE/NPB pathways are inducible in JS670, only growth on NPB led to upregulation of *npeA1* and *npeD*. The results indicate that *Burkholderia* sp. JS670 encodes two separate pathways involving dioxygenases for NPE/NPB and biphenyl degradation. The similarity of NpeA1 to biphenyl dioxygenases raises the questions of what selective advantage is conferred upon JS670 to maintain both pathways. Additionally, the biphenyl pathway uses the side chain as a growth substrate, whereas the NPE/NPB pathway must avoid further metabolism of the side chain, so must not induce the enzymes for side chain metabolism. Further analysis of the JS670 genome will provide insight into such questions.

Both the biphenyl dioxygenase and the 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase have previously been reported to be crucial in determining the substrate specificity of the biphenyl degradation pathway (17). The divergence of NpeA1 and NpeD from homologs in the biphenyl catabolic pathway provides compelling evidence that the pathway provides *Burkholderia* sp. JS670 with a unique substrate range and thus previously unknown catabolic capabilities. More extensive testing of the substrate range of NpeA1 and NpeD will be required to reveal the metabolic potential of

the two enzymes and the fate of side chains that are released as metabolites via the NPE/NPB pathway.

Although previously reported to be produced by both plants (24, 25) and fungi (2, 10), production of 3NPA during biodegradation of NPE by *Burkholderia* sp. JS670 is the first example of bacterial production of the toxin. Such findings are intriguing given the toxicity of 3NPA to livestock (1, 4, 40, 42, 47). Bacterial synthesis of 3NPA from NPE may represent an important source of 3NPA in natural ecosystems.

Auxanography experiments with both *Burkholderia* sp. JS670 and the NPB/NPE dioxygenase mutant on different natural and synthetic analogs of NPE and NPB demonstrated the key role of NpeA1 in catabolism of several such analogs, including nitrostyrene and phenylethylamine. The NPE/NPB degradation pathway thus provides *Burkholderia* sp. JS670 with a strategy to obtain carbon from a variety of substituted mono-aromatic substrates by eliminating the bulky side chain and utilizing the remaining carbon skeleton for growth.

The elimination of the side chain without using it as a growth substrate in NPE/NPB metabolism appears unique among *meta*-cleavage pathways. *Meta*-cleavage pathways are most commonly used for aromatic compounds with bulky side chains, enabling elimination and subsequent metabolism of the side chain. Conversely, *ortho*-cleavage pathways are more commonly used for simple aromatic compounds. With biphenyl and ethylbenzene degradation, elimination of the side chains, benzoate and propionic acid respectively, are catalyzed by hydrolases. Benzoate and propionic acid are subsequently mineralized. Goncalves *et al* found that in *Rhodococcus* sp. strain RHA1, a separate operon for benzoate degradation was upregulated by growth on biphenyl, but not

ethylbenzene, indicating benzoate metabolism was induced by biphenyl metabolism (20). Additionally, *Burkholderia xenovorans* LB400 encodes two different benzoate degradation pathways, one of which is preferentially induced with growth on biphenyl (12). Thus, there are well-established pathways for benzoate metabolism in both *Rhodococcus* sp. strain RHA1 and *Burkholderia xenovorans* LB400 that are induced by growth on biphenyl, indicating further catabolism of the side chain after elimination. The NPE/NPB pathway encodes a metabolic strategy to acquire carbon and energy from compounds without metabolizing the side chain. Such a strategy might protect the bacteria from toxic side chains or merely remove side chains that are not biodegradable from the larger compound.

Several lines of evidence indicate that the NPE/NPB degradation pathway is similar to, yet different from, the biphenyl degradation pathway. (1) *npeA1* and *npeD* are similar to genes encoding biphenyl enzymes, but are only upregulated when grown on NPB. (2) The NPE/NPB and biphenyl pathways are inducible by both NPB and biphenyl. (3) A *meta*-cleavage enzyme catalyzes the opening of the aromatic ring in both pathways. (4) In both NPE/NPB and biphenyl degradation, the side chain is eliminated from the parent compound and not used as a growth substrate. (5) Finally, JS670*npeA1*::pVIK110 lost the ability to degrade NPE/NPB, yet maintained the ability to grow on biphenyl.

Thus, the results presented in this study enable description of NPE/NPB metabolism through a pathway that is proposed to be similar to that for biphenyl degradation. The side chain released from the NPE metabolism was identified as 3NPA, and *Burkholderia* sp. JS670 appears to use less than half of the NPE molecule as a

growth substrate. Further studies are required to rigorously identify the intermediates of NPE/NPB metabolism. Additionally, sequencing of *Burkholderia* sp. JS670 is currently being conducted to identify the genes encoding the NPE/NPB and biphenyl degradation pathways.

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CHAPTER 5

CONCLUSIONS

5.1 Thesis summary

The objective of this work was to determine the novel molecular and biochemical mechanisms for the metabolism of 3NTyr and NPE/NPB and to understand why 3NTyr and NPE do not persist in natural ecosystems. In chapter 2, qRT-PCR and insertional mutagenesis revealed that *denA* is the gene necessary for encoding the HNPA denitrase involved with 3NTyr degradation. Furthermore, the function of a previously uncharacterized flavin monooxygenase in *Bordetella bronchiseptica* RB50 was identified as an HNPA denitrase. In chapter 3, analysis of soil and water samples revealed that 3NTyr degradation is widespread among sampled sites. Additionally, comparison of 16S rRNA sequences from the 3NTyr degrading isolates revealed that the ability to metabolize 3NTyr is common to *Actinobacteria* and several classes of *Proteobacteria*. Comparison of DenA sequences in select 3NTyr-degrading isolates allowed for further identification of conserved sequence motifs in such enzymes. Finally, in chapter 4, respirometry, observation of dioxygenase activity, detection of the *meta*-cleavage compound and insertional mutagenesis enabled description of the biodegradation pathway for NPE/NPB, which is the first report of degradation of a natural nitroaliphatic compound. Furthermore, qRT-PCR confirmed the upregulation of *npeA1* and *npeD*, which, coupled with growth studies with JS670*npeA1*::pVIK110, demonstrated that both

genes encode enzymes that are involved in NPE degradation, and that *npeA1* is specifically required for biodegradation of NPE and NPE homologs.

5.2 Summary of Dissertation Research Findings

5.2.1 Discovery of *denA* in *Variovorax* sp. JS669

In chapter 2, the isolation of the 3NTyr-degrading bacteria *Variovorax* sp. JS669 enabled further study into the molecular biology of *denA*, the gene encoding the HNPA denitrase. Although the HNPA denitrase had been previously described (9), this report is the first description of *denA*. The discovery of *denA* permitted characterization of conserved sequence motifs present in both the gene and the encoded protein, as well as identification of the HNPA denitrase as a class A flavin monooxgenase. The JS669 HNPA denitrase is the first biochemically-characterized enzyme in its clade. Additionally, comparison of the HNPA denitrase with other enzymes capable of denitration, dechlorination and defluorination yielded discovery of several conserved residues among the group of enzymes that catalyze such reactions.

5.2.2 Phylogenetic Comparison of 16S rRNA and *denA* Genes among 3-NTyr-degrading bacteria

The research in chapter 3 revealed widespread 3NTyr degradation and isolation of phylogenetically diverse 3NTy-degrading bacteria. Bacterial isolates representing four distinct 16S rRNA phylogenetic groups were identified from a variety of different habitats, although the majority of isolates were obtained from several different agricultural sites in Georgia and Virginia. All of the isolates degraded 3NTyr and the overwhelming majority of the isolates released nitrite HNPA, implying that such isolates used the previously described 3NTyr degradation pathway (9). The observation that 3NTyr disappeared in enrichment cultures from every sampled site, coupled with the facile isolation of 3NTyr-degrading bacteria revealed the ubiquitous distribution of the ability to degrade 3NTyr within various ecosystems.

The phylogenetic relationships among DenA of 3NTyr-degrading isolates are also described in chapter 3. All of the identified DenA enzymes group in a clade among related Class A flavin monooxygenases. Such sequence similarity enabled the development of DenA specific probes that amplified *denA* in 50% of the tested isolates. The DenA probes are non-degenerate and in part target a key conserved motif among flavin monooxygenases. Such probes enabled the identification of additional *denA* sequences in isolates that had not previously revealed such genes as well as a second gene, *denB*, in *Variovorax* sp. JS171 that probably encodes the HNPA denitrase required for 3NTyr degradation.

5.2.3 The NPE/NPB degradation pathway in *Burkholderia* sp. JS670.

In chapter 4 the degradation pathway for NPE/NPB in *Burkholderia* sp. JS670 is revealed. Initial observations during NPE/NPB metabolism indicated that a dioxygenase is involved during growth on NPE/NPB and the accumulation of the putative NPE/NPB side chain in the growth media. The predicted NPE/NPB pathway was confirmed by oxygen-uptake experiments, transient accumulation of the *meta*-cleavage compound and identification of 3NPA/3NBA.

The genes encoding the alpha subunit of the NPE/NPB dioxygenase (*npeA1*) and the hydrolase involved with NPE/NPB degradation (*npeD*) are also described in chapter 4. Both genes are upregulated in cells grown on NPB. An insertional mutant in *npeA1* confirmed the essential role of NpeA1 in NPE/NPB degradation. Additionally, both JS670 and the Δ*npeA1*::JS670 construct were used to identify other substrates that are metabolized by the NPE/NPB pathway. Based on such experiments, it is apparent that the NPE/NPB degradation pathway is useful for metabolism of analogs of NPE/NPB. Thus the data reveal that the NPE/NPB pathway might represent a more universal metabolic pathway by which JS670 removes side chains from aromatic compounds to obtain carbon for growth.

5.3 Broader Implications of Research

Identification of the gene encoding the *Variovorax* sp. JS669 DenA in chapter 2 is the first description of such a gene and as such is of crucial importance in the understanding of 3NTyr catabolism. Flavin monooxygenases are well-studied enzymes, yet DenA represents a previously uncharacterized clade of enzymes. The study of DenA permitted the characterization of a previously uncharacterized enzyme and will enable further such endeavors.

The HNPA denitrase in *Variovorax* sp. JS669 represents a novel clade of Class A flavin monooxygenases. Several enzymes with sequence similarity to *denA* were putatively annotated in databases as flavin monooxygenases, however biochemical testing of the enzyme in *Bordetella bronchiseptica* RB50 revealed that it is a functional HNPA denitrase. Although the other tested organisms with *denA*-like genes in the HNPA denitrase clade do not degrade 3NTyr or HNPA, it is possible that they catalyze similar reactions. Specifically, the proteins within the HNPA denitrase clade that do not catalyze HNPA denitration may instead be involved with denitration of 3NTyr or HNPA homologs, including 3-nitrotyramine. Moreover, the DenA clade of flavin monooxygenases may represent a group of enzymes that enable denitration of amino acids, such as 4-nitrotryptophan, or other compounds similarly altered via nitration. The research findings presented in chapter 2 thus advance the understanding of flavin monooxygenases and enable the re-annotation of several putative enzymes via biochemical characterization.

3NTyr is ubiquitously found at sites of infection and inflammation; yet despite an enormous amount of research about 3NTyr (3, 7, 8, 11) there is only one report of its complete metabolism (9). Although HNPA denitration has been previously described as part of the microbial biodegradation pathway for 3NTyr (9), until this report no specific genes in the 3NTyr degradation pathway have been characterized. As HNPA is an intermediate in both 3NTyr and 3-nitrotyramine degradation (9, 13), DenA may be a shared enzyme between the two pathways and thus may represent an evolutionary link between the 3NTyr and 3-nitrotyramine metabolic pathways.

As described in chapters 2 and 3, nitration via activity of reactive nitrogen species is a potential result of host defense mechanisms; it is thus plausible that DenA represents a mechanism to evade such chemical defenses. Increased NO and reactive nitrogen species production in hosts during initial stages of infection and inflammation are typical, and yet organisms capable of 3NTyr degradation may be able to evade the toxicity of the nitrated compounds generated via reactive nitrogen species. Such a strategy would enable 3NTyr-degrading bacteria to invade host species or decrease the concentration of 3NTyr and analogs in nitrated proteins, thereby providing the 3NTyr degrader with the ability to infect a host as an opportunistic pathogen or as a symbiont depending on the situation.

Research in chapter 4 is the first to describe bacterial growth using a natural nitro compound with an aliphatic side chain, and might represent another mechanism by which bacteria avoid host chemical defenses. Although the ecological role for NPE is unknown, the production of NPE along with several insecticide and anti-predation compounds implies that NPE might represent another plant defense. Bacterial degradation of NPE

could thus represent a method by which bacteria avoid plant defenses, or conversely enable the bacteria to obtain carbon from a secondary plant metabolite and generate another toxin, specifically 3NPA.

Previously reported sources for 3NPA include a variety of fungi (2), leguminous plants (1, 5, 6) and at least one report of 3NPA synthesis by a fungal endophyte (4). The research presented in chapter 4 clearly identified another source for the toxin. Although 3NPA is produced by the plants and fungal endophytes, it is still possible that the toxin is actually produced by bacteria acting on the NPE produced by plants. It is thus possible in agricultural ecosystems, that bacteria degrading NPE are producing the high levels of 3NPA that are blamed for congestive heart failure, ataxia and issues with fetal development including neonatal death in livestock (10, 12).

Finally, the NPE/NPB degradation pathway is a novel metabolic strategy by bacteria to obtain carbon from a compound by elimination of a bulky side chain. This method might enable bacteria to obtain carbon and energy from more diverse compounds, thereby providing a growth advantage to microorganisms possessing the NPE/NPB degradation pathway.

5.4 Future work

The biological substrate for the DenA-like enzymes remains unknown. The natural occurrence of a vast number of analogs for 3NTyr and HNPA implies that there may be other similar denitration reactions. Additionally, the substrate range for DenA is

unknown, indicating that DenA might denitrate analogs of HNPA. Purification of DenA will enable research to study the substrate and inhibitor range of DenA, as well as further advance the biochemical study of the enzyme. Furthermore, determination of the physiological substrates for other DenA and DenA-like enzymes will provide insight into the role of HNPA denitration and the selective advantage conveyed by such metabolism.

Sequencing of *Variovorax* sp. JS669 and subsequent annotation will facilitate further discovery of the unique metabolism of this microorganism. Although the biodegradation pathway for 3NTyr has been determined (9), *denA* is the only gene from the pathway that has been identified. Complete annotation of the genome of *Variovorax* sp. JS669 will permit discovery of the genes that encode the decarboxylase and deaminase, which are necessary for 3NTyr degradation. Both the decarboxylase and deaminase involved with 3NTyr degradation enable catalysis with novel substrates, and are not involved with tyrosine degradation. Furthermore, it will be of interest to determine whether 3NTyr-degrading bacteria can also degrade 3-nitrotyramine, as well as other nitrated compounds such as 4-nitrotryptophan. Finally, only one other *Variovorax* sp. genome has been sequenced and partially annotated, *Variovorax paradoxus* S110 (Accession numbers NC_012791 and NC_012792 for chromosomes 1 and 2, respectively). Thus, it will be interesting to complete annotation of *Variovorax* sp. JS669 in order to check for other genes that might encode novel catabolic enzymes and compare the genomes of JS669 and *Variovorax paradoxus* S110.

As mentioned in chapter 2, the degenerate primers amplify *denA* sequences in 50% of the tested 3NTyr-degrading isolates, which yields questions as to the divergence of DenA among the isolates. The remaining isolates might have sufficiently divergent

denA, such that the probes do not detect the genes. Another possibility is that there are additional pathways for 3NTyr degradation. The vast majority of isolates produced nitrite from HNPA, however there were several isolates that degraded 3NTyr but did not release nitrite from HNPA. It is thus possible that such isolates are either utilizing the nitrite released from HNPA, or are using an alternative metabolic pathway for 3NTyr degradation. Further testing of the 3NTyr-degrading isolates will be required to distinguish among the alternative hypotheses.

The development of DenA-specific probes will facilitate the identification of more 3NTyr-degrading isolates and *denA* genes in various ecosystems. Identification and analysis of additional *denA* genes will provide insight into the evolution and diversity of such genes. Further study of DenA will enable identification of more members of the denitrase clade and reveal the substrate range of the enzymes. Determining the role of 3NTyr degradation in pathogenesis, host selection, or niche establishment of 3NTyr-degrading bacteria will reveal the ecological impact of 3NTyr metabolism. Additionally, determination of whether 3NTyr degraders use such metabolism to protect from auto-nitration of their proteins will provide further insight into the role of 3NTyr metabolism.

Research in chapter 4 revealed the biodegradation pathway for NPE and NPB in *Burkholderia* sp. JS670, however there is still much to understand about NPE metabolism and the enzymes responsible for degradation. First, it is unknown whether the ability to degrade NPE is widespread or specific to bacteria occupying areas surrounding plants that produce NPE. Second, it is plausible that endophytes of plants, such as vetch, that produce NPE can also degrade NPE, and should thus be investigated for the ability to metabolize NPE. Finally, purifying the enzymes required for NPE/NPB degradation and

determining their substrate ranges will enable further understanding about the roles of such enzymes. As demonstrated in chapter 4, the NPE/NPB degradation pathway, specifically NpeA1, is also used for metabolism of NPE analogs and other secondary plant metabolites. Determining the complete spectrum of natural and synthetic compounds degradable through the NPE/NPB pathway will reveal the capabilities of the NPE/NPB enzymes, potentially enabling their manipulation and use for the degradation of more recalcitrant, synthetic compounds or development of green chemistries for the synthesis of novel compounds.

The discovery of 3NPA production by a bacterium provides for a new direction of study into the synthesis of the 3NPA toxin. The discovery that 3NPA is produced by NPE-degrading soil microbes isolated from near tomato plants prompts questions as to whether similar microbes might be isolated from near other NPE-producing plants such as orchids or Wright's gardenias. Isolating endophytic bacteria capable of NPE metabolism from inside plants, such as vetch and locoweed, associated with 3NPA production will provide further insight into the ecological impact of NPE degradation/3NPA synthesis in niche establishment for such bacteria.

Completing the sequencing and annotation of *Burkholderia* sp. JS670 will permit the study of the genes encoding the metabolic pathways encoded by the organism, including the NPE/NPB pathway and a second biphenyl degradation pathway. It is intriguing that JS670 appears to have two very similar degradation pathways for NPE/NPB and biphenyl, or at least two similar dioxygenase α-subunits. First, why does JS670 maintain two such similar enzymes or metabolic pathways? Second, what evolutionary mechanism provided for the development of the second pathway? Gene

duplication is one potential way in which the two pathways might have evolved, but other evolutionary processes for obtaining both pathways are possible.

5. 5 Final thoughts

Identification of novel microbial catabolism, such as that for NPE/NPB is an effective means to understanding the breadth of microbial-mediated degradation and the substrate range of such catabolic enzymes. Because there are numerous natural and synthetic analogs of both natural and anthropogenic nitro compounds (14), deciphering the biodegradation pathway of one compound provides insight into metabolism of many additional compounds. Furthermore, it is likely that enzymes for the metabolism of natural nitro compounds have provided the basis for the biodegradation of many synthetic chemicals, thus determination of the metabolic pathway for natural nitro compounds also provides the basis for understanding the evolution of pathways for the biodegradation of anthropogenic compounds. The determination of biodegradation pathways for natural nitro compounds provides the basis for the development of biodegradation pathways to aid in bioremediation of nitro compounds and synthesis of novel compounds. Finally, identification of the genes encoding the key enzymes DenA, NpeA1 and NpeD; and substrate ranges of such enzymes in natural nitro compound biodegradation pathways enables the use of such enzymes in hybrid biodegradation pathways.

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